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Ultrastructural observation on sterilization of melittin

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The effects of melittin on growth and bacteriostasis of four pathogens were extensively investigated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The results indicated that the melittin had a marked bacteriostatic effect on the four pathogenic bacteria. Among these, *E. cacotowora* was influenced most powerfully and quickly, the yeast and *F. fulva* were the second, and the *S. aureus* was inhibited by a low concentration but was killed by a high concentration. It was observed in the experiments that melittin killed pathogenic bacteria in three ways. One was by pore formation. After integrating melittin into the plasma membrane, a vacuole was formed then penetrated, resulting in bacterial content leakage. The vacuole also experienced plasmolysis and the growing cavity destroyed the membrane. A third effect was the formation of vacuoles in the cells which induced the pycnosis of the cytoplasm resulting in a cell death. The mechanism of melittin bacteriostasis was the result of integrating melittin with phospholipod double layers of the plasmalemma and the endomembranes.

Melittin, pathogens, ultrastructural alterations, bacteriostasis mechanism

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The antimicrobial peptides, cecropins, were originally isolated from silkworms by the Swedish scientist Human G. in 1980 [1]. Pharacologists regarded them as possible low resistence as antibiotics [2]. Various studies have been concerned with the antimicrobial mechanism. Benachir considered that the affinity of melittin for phosphatidylcholine vesicles was responsible for the increasing melittin-induced leakage from cholesterolcontaining membranes, and an all-or-none hypothesis was proposed [3]. The Shai-Matsuzaki-Huang (SMH) model proposed that the interaction of the peptide with the membrane was followed by the displacement of lipids [4–8]. Five hypotheses concerning the possible mechanisms for antimicrobial peptide action [3] were summarized in *Nature*. However, the mechanisms of antimicrobial peptide action mentioned above are based on the study on cecropins, with related few reports concerning other varieties of antimicrobial peptides.

Melittin is an insect antimicrobial peptide with an especially sterilization capacity. Melittin has broad-spectrum, fast-acting and highly effective inhibitory effects on both pathogenic and agricultural microorganisms, which demonstrated its application potential as a biological pesticide [9]. There has been no systematic research regarding the mechanism of melittin action at the ultrastructural level, nor has any direct physical evidence been confirmed. We studied the ultrastructural changes of bacteria, yeast and fungi after their interaction with melittin, and tried to find more visual evidence in order to both confirm the hypothesis concerning physiological and biochemical aspects, and also explore the mechanism of the interactions between melittin and various pathogens.

1 Materials and methods

1.1 Materials

1.1.1 Strains

Erwinia carotovora and *Fulvia fulva* were obtained from the Chinese Agricultural Academy of Sciences and Shen-

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yang Agriculture University. *Staphylococcus aureus* and *Escherichia coli* were obtained from CMCC and GS115 from TaKaRa.

1.1.2 Melittin sample preparation

Melittin prepared as 0.25 mg mL^{-1} stock solution in 0.9% NaCl was purchased from Baiyunshan Pharmaceutical Company in China.

1.2 Methods

1.2.1 Microbial culturing and growth curves

Sample groups in LB media were either composed of 2 mL melittin (0.25 mg mL^{-1}) or 2 mL sterilized water (blank control). *Erwinia carotowora, Staphylococcus aureus,* GS115 and *Fulvia fulva* in the logarithmic growth stage were each added into both groups (0.4 mL) which were cultured synchronously. A_{600} was determined spectrophotometrically after respectively being cultured for 0, 1, 2, 4, 8 and 12 h. The percentage of antibacterial potency was calculated according to the equation (A_{600} of 0 hour−A_{600} of x hour)/A_{600} of x hour×100% (here, x indicates definite incubation hours). Data obtained in this study were all obtained after being repeated 3 times. Differences between groups were analyzed using SPSS 10.0，which was expressed as the average± standard error ($\overline{x}$ ±SE).

1.2.2 Sample preparation

Pathogen at logarithm growth was added to LB media with melittin (0.125 mg mL^{-1} and 0.25 mg mL^{-1}), and swayedly cultured. After being cu1tured for respectively 1, 4, 8 and 12 h, samples were harvested for observation. The control group was prepared and observed following the same procedure described above.

1.2.3 Electron microscopy observation

The concentrated pathogens were fixed with glutaraldehyde for at least 2 h. The samples were prepared according to the methods of sample preparation for transmission electron microscope scanning [10].

2 Results and analysis

2.1 Inhibitory effects of melittin on different pathogens

After incubation for 24 h, the addition of melittin rapidly affected the growth of four kinds of pathogens, while the control group grew at a normal rate. In order to compare the sensitivities of different strains to melittin, the changes of A_{600} Value were tested, and the percentages of antibacterial efficiency were calculated. The effect of melittin on *pathogens* is shown in Figure 1. Compared with the control group, the A_{600} value of *Erwinia carotovora* with melittin decreased from 0.24 to 0.01 within 1 h and remained at the value of 0.01 for more than 12 h, which changed the sterilization percentage to 95.8%. However, the A_{600} value of *Staphylococcus aureus* with melittin decreased to 0.10 within 1 h and decreased to 0.04 4 h later, which changed the sterilization percentage to 85.1%. But 8 h later, the A_{600} value was increased to 0.07, decreasing the percentage to 60.3%, which meant that melittin exhibited inhibitory action solely at low concentrations. When the concentration of melittin doubled, there was no growth recovered with 95% inhibition. The inhibitory percentage of GS115 with melittin was observed after 1 h at 25.2%, 4 h at 50.0% and 8 h with 92.2%. For *Fulvia fulva*, the inhibitory action was 19.4% after 1 h, 46.6% after 4 h and 94.1% after 12 h. The growth of both GS115 and *Fulvia fulva* was not recovered after 24 h.

It was suggested that melittin might have directly killed *Erwinia carotovora,* GS115 and *Fulvia fulva* rather than inhibiting the growth, with the rapid inhibitory action of melittin on *Staphylococcus aureus* exclusively at its lowest concentration.

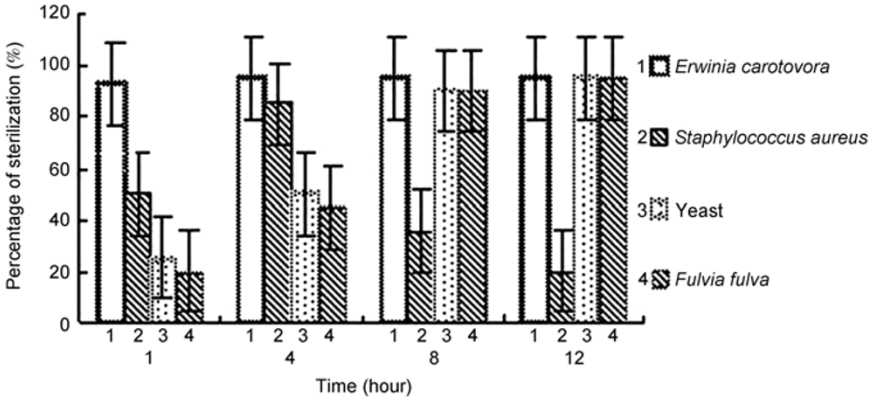

Figure 1 Comparison of inhibitory percentage of melittin on pathogens.

2.2 Electron microscope observation of bacteria

2.2.1 Ultrastructure changes of Erwinia carotowora in response to the bacteriostatic action of melittin

The healthy cell membrane of *Erwinia carotowora* is typically intact and slick, the cell structure is compact and inerratic, and the cytoplasm is well-proportioned. By contrast, the configuration of melittin affect *Erwinia carotowora* was transfigured after 4 h, the cytoplasm was no longer well-proportioned, contracting and gathering, vacuoles appeared in the inner of the cell (Figure 2A), as well as holes in the cell membranes (Figure 2B). Each hole pierced through the cell membrane from the inside to the outside (Figure 2C). With the increase of intensity, the holes gradually expanded. The proliferation of holes resulted in the fragmentary of membranes, and leaking of cell contents. Ultimately, the pathogens cracked and died (Figure 2A).

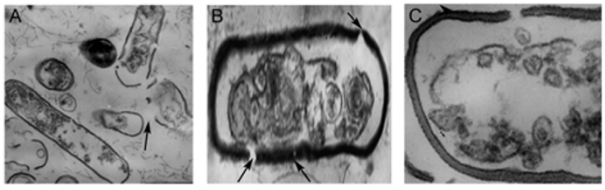

Figure 2 Interior structure of *Erwinia carotowora* under a transmission electron microscope. A, *Erwinia carotowora* affected by melittin for 1 h (×8000); B, *Erwinia carotowora* affected by melittin for 1 h (×300000); C, *Erwinia carotowora* affected by melittin for 4 h (×500000).

2.2.2 Ultrastructure changes of Staphylococcus aureus in response to the bacteriostatic action of melittin

Healthy *Staphylococcus aureus* is a well-stacked ball with a smooth surface and compact, well-propotioned cytoplasm (Figure 3A). Following 4 h under the influence of melittin, small vacuoles were seen on the cell membrane of *Staphylococcus aureus*, with the vacuoles looking like bubbles gradually extended towards both sides of membrane, with adjacent vacuoles connected to form larger vacuoles between the cell wall and the cytoplasm, a further expanded (Figure 3B). The cytoplasm sharply contracted, accompanied by plasmolysis, which caused cell death (Figure 3C).

2.2.3 Scanning electron microscope observation of Staphylococcus aureus and Erwinia carotowora

The typical cell membrane of *Staphylococcus aureus* and

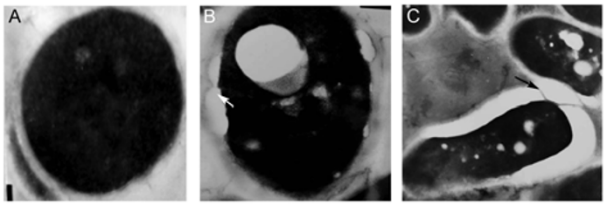

Figure 3 Interior structure of *Staphylococcus aureus* under a transmission electron microscope. A, healthy *Staphylococcus aureus* (×20000); B, *Staphylococcus aureus* affected by melittin for 1 h (×20000); C, *Staphylococcus aureus* affected by melittin for 4 h (×10000).

Erwinia carotowora is bacilliform, slick and full when observed by a scanning electron microscope (Figures 4A and 5A). Following being affected by melittin for 4 h, the configuration was deformed, the surface became rough, toothed and then began to decompose. The remanet mass was observed and all pathogens died (Figure 4B). The cell contents leaked out, which buried the cell in kytoplasm (Figure 5B).

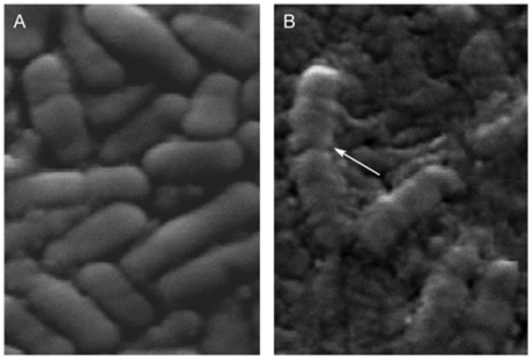

Figure 4 Exterior configuration of *Erwinia carotowora* under a scanning electron microscope. A, normal *Erwinia carotowora* (×10000); B, *Erwinia carotowora* affected by melittin for 1 h (×10000).

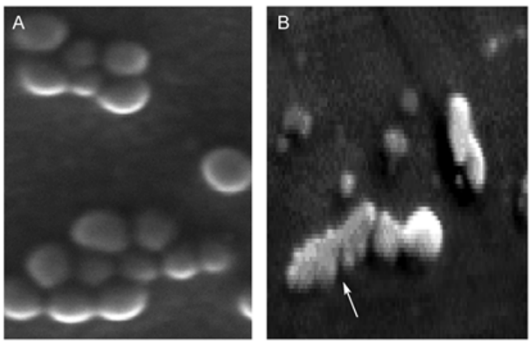

Figure 5 Exterior configuration of *Staphylococcus aureus* under a scanning electron microscope. A, healthy *Staphylococcus aureus* (×10000); B, *Staphylococcus aureus* affected by melittin for 1 h (×10000).

2.3 Electron microscope observation of yeast GS115

Healthy yeast looks like an energetic ball with a smooth surface, compact and well-propotioned cytoplasm (Figure 6B). Many vacuoles appeared at the cell membrane (Figure 6A), which gradually expanded both inside and outside, and then the membrane broke though. Inward expansion made cytoplasm scatter to form a cavity. Cytoplasm and various enzymes were leaked into the area between the the membrane and the cell wall, which disintegrated the cell wall (Figure 6C). The proliferating holes made the cell membrane and the cell wall no longer viable with the cell content lost, cell cracked and dead (Figure 6D).

Healthy yeast is spherical with a smooth surface (Figure 7A). After 4 h of being affected with melittin, the cell sur-

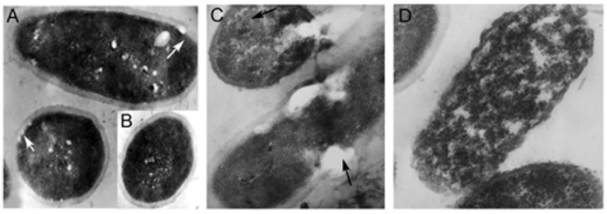

Figure 6 Interior structure of yeast under a transmission electron microscope. A, yeast affected by melittin for 1 h (×10000); B, healthy yeast (×6000); C, yeast affected by melittin for 4 h (×10000); D, yeast affected by melittin for 8 h (×10000).

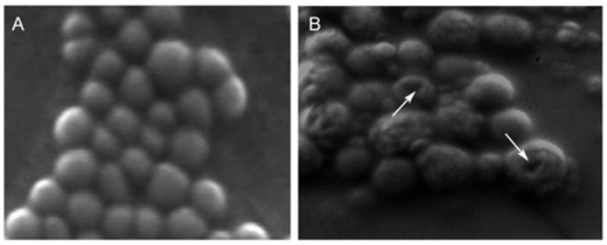

Figure 7 Exterior configuration of *Erwinia carotowora* under a scanning electron microscope. A, healthy yeast (×10000); B, yeast affected by melittin for 4 h (×10000).

face become quite rough, and disintegrated. Holes which extended from the inner to the outer membrane were observed with SEM, the contents leaked, and the cell died (Figure 7B).

2.4 Electron microscope observation of fungi

The cross section of healthy spores of *Fulvia fulva* is nearly round, inerratic; the cell wall is thick, the raphe-like tuber in its tegument is well-proportioned; the cytoplasm is regular (Figure 8A). By contrast, fuugi of the group affected by melittin became crimped and distorted, the cytoplasm contracted, the cavum emerged, the cytoplasm and cell wall separated, and the tuber disappeared (Figure 8B).

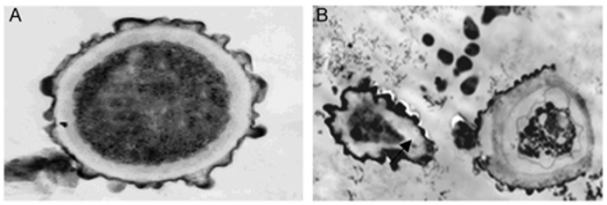

Figure 8 Interior structure of *Fulvia fulva* under a transmission electronmicroscope. A, healthy *Fulvia fulva* (×40000); B, *Fulvia fulva* affected by melittin for 12 h (×30000).

Contrasting with the control, the pathogens configuration was markedly affected by melitiin after 24 h. The involved fungus was distorted, the cell surface was irregular, the granule in it disappeared, and the exterior crimped and partially split (Figures 9A and B).

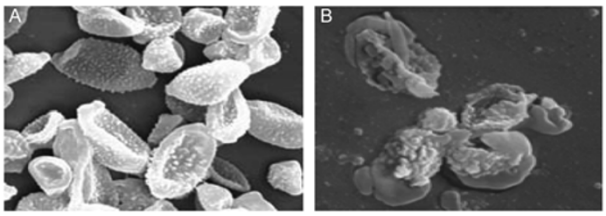

Figure 9 Exterior configuration of *Fulvia fulva* under a scanning electronmicroscope. A, healthy *Fulvia fulva* (×9000); B, *Fulvia fulva* affected by melittin for 12 h (×9000).

3 Discussion

3.1 Analysis of membrane punching hypothesis

According to the results obtained from biochemistry and molecular biology, 5 kinds of hypothesis were proposed for the possible sterilization mechanism of antimicrobial peptide [4]. We observed the entire process under SEM and TEM for the first time, in the course of which the pathogens deformed, the membrane punched, the cell walls disintegrated, with the pores increased in size and number, the cytoplasm leaked, and the cells died. The results confirmed the view of melittin punching in the membrane. The interaction of melittin with yeast and bacteria were very clear. In our experiments we also observed the formation of holes with clear edges in the membrane. Melittin permeates the cell wall to affect the membrane. Initially, a vacuole with a diameter of approximately 30 nm formed in the center of the cell membrane. After the diameter of plasm side constantly expanded up to 70 nm, the vacuole penetrated the cell wall from inside to outside, forming a clear structure analogous to an electric drill hole. We selected four common pathogens, including *Staphylococcus aureus, Erwinia carotowora,* yeast and *Fulvia fulva,* to research their interaction with melittin under an electron microscope. From the changes of ultrastructure we observed, it was concluded that melittin penetrated the cell wall and affected the cell membrane and the cytoplasm, in which enzymes and acceptors were included. There are several possible models for the interaction of melittin with the membrane: drilling holes, breaking out, or rolling up, which were observed in different strains. The influence of melittin on the cytoplasm led it to contract, and then triggered apoptosis. There were differences in the mechanism between species in the process of melittin's action on the membrane and the cytoplasm, which might be derived from minute differences in membrane structure. The cell walls of such fungi as *Fulvia fulva* may be broken down after interaction with melittin. It was also discovered that the membrane punching mechanism also involved the barrel-stave model and the toroidal model [11], which may require further elucidation.

3.2 Interrelation between the action and speed or degree in bacteriosis of melittin

The study above indicated that melittin had different kinds

of actions on various pathogens. It was observed that there were differences in the strength of the interaction with melittin among the different pathogens. The A_{600} value of *Erwinia carotovora* with melittin was decreased from 0.24 to 0.01 within 1 h and remained at 0.01 until 12 h later, which changed the percentage of sterilization to 95.8%. However, the A_{600} value of *Staphylococcus aureaus* with melittin decreased to 0.10 within 1 h and to 0.04 4 h later, which changed the percentage of sterilization to 85.1%. But 8 h later, the A_{600} value was increased to 0.07, which changed the percentage to 60.3%, signifying that inhibited solely at low concentration. When double concentration were taken, there was no growth recovered with an inhibory percentage of 95%. The inhibitory percentage of melittin on GS115 was 25.2% after being treating for 1 h, 50.0% after 4 h and 92.2%. after 8 h. The same influence on *Fulvia fulva* 19.4% after 1 h, 46.6% after 4 h and 94.1% after 12 h. Both GS115 and *Fulvia fulva* had no recovery growth after being incubating for 24 h. There was apparently a direct relationshio between the active strength and the time course. The "Punching model" may lead to various bacteriostasis quickly and effectively [11]. The "Plasmolysis model" was weak, while the "Cytoplasmic-cavity model" was strong but slow. Whether or not the observed phenomenon was universal requies experimental conformation.

From the above comprehensive study, we propose that all of the interactions between melittin and pathogens directly influence on the cell membrane [3].

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