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Effects of cinnamaldehyde on *Escherichia coli* and *Staphylococcus aureus* membrane

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ABSTRACT

Gram-negative *Escherichia coli* (ATCC 8735) and Gram-positive *Staphylococcus aureus* (ATCC 3101) were selected as model bacteria to determine the antimicrobial mechanism of cinnamaldehyde. Several techniques were utilized to investigate the effects of cinnamaldehyde on food-borne bacterial membranes. The ultraviolet (UV) absorption and electrical conductivity of the culture supernatant were used to determine membrane integrity. β-Galactosidase activity was determined to detect inner membrane permeability. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed to observe bacterial morphology. Samples from both strains exposed to cinnamaldehyde showed higher UV absorptions, conductivity values, and β-Galactosidase activities compared with the control group and displayed a rapid rise trend. Thereafter, the values stabilized at a relatively steady state. SEM shows that treated *E. coli* and *S. aureus* cell samples exhibited rough cell membranes with particulate matter, and some of the *S. aureus* cells split due to deep wrinkle formation and distortion, unlike the control group. TEM shows that the bacteria treated with cinnamaldehyde exhibited numerous abnormalities, including cytoplasmic membrane separation from the cell wall, cell wall and cell membrane lysis, cytoplasmic content leakage, cytoplasmic content polarization, cell distortion, and cytoplasmic content condensation. These results indicate that bacterial cell morphology, membrane integrity, and permeability are damaged when the *E. coli* and *S. aureus* cells are exposed to the minimum inhibitory concentrations of cinnamaldehyde (0.31 mg/mL). In addition, the higher the cinnamaldehyde concentration, the more serious the bacterial membrane damage is.

1. Introduction

The use of natural antimicrobials instead of traditional preservation techniques, such as heat treatment, dehydration, and chemical preservatives, has gained popularity in recent years because consumers increasingly prefer food processed through milder preservation techniques because food processed this way have enhanced natural appeal and perceived nutritional quality. Spices and their extracts are generally recognized as safe because of their traditional uses without any documented detrimental effects (Newton, Lau, & Wright, 2000; Shan, Cai, Brooks, & Corke, 2007). Cinnamaldehyde has been isolated from cinnamon essential oils from the 19th century and is mainly used to impart cinnamon fragrance to medical products, cosmetics, and perfumes (Bickers, Calow, Greim, & Hanifin, 2005). In recent years, cinnamaldehyde has been reported to have a broad spectrum of antimicrobial activity (Gallucci et al., 2009; Inouye, Takizawa, & Yamaguchi, 2001; Palaniappan & Holley, 2010; Pei, Zhou, Ji, & Xu, 2009; Sanla-Ead, Jangchud, Chonhenchob, & Suppakul, 2012; Tunc, Chollet, Chalier, Preziosi-Belloy, & Gontard, 2007), as verified by our previous study (Ye et al., 2013). Studies on the antibacterial mechanism of cinnamaldehyde are lacking, which limits the development and utilization of cinnamaldehyde as food preservative. The cytoplasmic cell membrane is the target of many antibacterial agents. The interactions of bacterial membranes with biocides frequently cause fundamental changes in both membrane structure and function (Denyer, 1990). Several methods were used to determine the effects of cinnamaldehyde on the food-borne bacterial membrane. The ultraviolet (UV) absorption and the electrical conductivity of the culture supernatant were used to determine membrane integrity. β-Galactosidase activity was used to detect inner membrane permeability. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) observations were used to determine bacterial morphology.

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2. Materials and methods

2.1. Reagents and strains

Cinnamaldehyde (98%) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Cinnamaldehyde was dissolved in dimethyl sulfoxide to 5 g/L concentration under sterile conditions, and the solution was stored at 4 °C. The food-borne microbial strains, Gram-negative *Escherichia coli* (ATCC8735) and Gram-positive *Staphylococcus aureus* (ATCC3101), were obtained from the Institute of Zoonosis of Jilin University. Active cultures were generated by inoculating thawed microbial stock suspensions into beef-extract peptone media, which were incubated overnight at 37 °C with shaking at 135 r/min. O-Nitrophenyl-β-D-Galactopyranoside (ONPG) was purchased from Sigma–Aldrich Inc. (St. Louis, USA).

2.2. Instruments and equipment

The instruments used include UV visible spectrophotometer (UV-2550, Shimadzu Corporation, Kyoto, Japan), DDSJ-308A conductivity meter (Shanghai Ray Magnetic Instrument Factory Co., Ltd., Shanghai, China), vacuum freeze drier (ES-2030, Hitachi, Kyoto, Japan), ion sputtering apparatus (E-1010, Hitachi, Kyoto, Japan), scanning electron microscope (S-3400N, Kyoto, Japan), and transmission electron microscope (H-7650, Kyoto, Japan).

2.3. UV absorption determination

The experiments were conducted based on a published method (Chen & Cooper, 2002), as follows. The bacterial suspension was centrifuged at 5000 r/min for 10 min and was washed thrice by using phosphate buffered saline (PBS, pH 7.4). The suspension was diluted to the test concentration, which made the absorbance of the bacterial suspension at 630 nm (A_{630nm}) reach 0.5 ± 0.02. The bacterial suspension was separated into several flasks. Cinnamaldehyde was added to each flask, except to the control. In our previous report (Ye et al., 2013), cinnamaldehyde was found to have the same antimicrobial activity toward *E. coli* and *S. aureus*, and the minimum inhibitory concentrations (MICs) are both 0.31 mg/mL. Three series of tests were designed in this study for cinnamaldehyde: MIC (0.31 mg/mL), 2MIC (0.63 mg/mL), and 4MIC (1.25 mg/mL). A 1.5 mL sample was removed from the flasks at 0, 1, 2, 4, 6, and 8 h of incubation at 37 °C. The samples were immediately filtered using 0.22 μm syringe filters to remove bacteria. The optical density (OD) of the supernatant at 260 nm was recorded using UV visible spectrophotometer. All tests were performed in triplicate.

2.4. Conductivity determination

Cellular leakage from the bacteria was measured by detecting electrolyte leakage into the incubation medium by using a conductivity meter, based on a previously published method (Lee, Choi, & Cho, 1998; Lee, Duke, Birk, Yamamoto, & Duke, 1995). The bacterial suspension was centrifuged at 5000 r/min for 10 min and was washed thrice by using 0.85% NaCl. The suspension was diluted, separated into several flasks, and added with cinnamaldehyde in a similar fashion as that of UV absorption determination.

A 5 mL sample was removed from each flask at 0, 1, 2, 4, 6, and 8 h of incubation at 37 °C. filtered using 0.22 μm syringe filters to remove bacteria. Supernatant conductivity was determined using a conductivity meter. All tests were performed in triplicate.

2.5. β-Galactosidase activity determination

Inner membrane permeability was determined by measuring β-Galactosidase activity in *E. coli* or *S. aureus* by using ONPG as substrate (Falla, Karunaratne, & Hancock, 1996; Lehrer et al., 1989). Logarithmic phase bacteria were washed thrice by using 0.05 mol/L NaH_2PO_4 phosphate buffer. The bacteria were then diluted to the test concentration, which made the absorbance of the bacterial suspension at 600 nm (A_{600nm}) reach approximately 0.2. Different amounts of cinnamaldehyde were added to the test groups at time 0, except for the control group. About 10 mL of the samples was removed from the flasks at 0, 1, 2, 4, 6, and 8 h of incubation at 37 °C. The samples were then immediately centrifuged at 10,000 r/min for 15 min at 4 °C. Approximately 1 mL of supernatant was transferred to a flask. Subsequently, 4 mL of 0.05 mol/L ONPG was added. The solution was made to react in a water bath at 37 °C for 40 min. Finally, 5 mL of 0.5 mol/L Na_2CO_3 was added to terminate the reaction. After 1 min, the production of o-nitrophenol over time was monitored spectrophotometrically at 420 nm. All tests were performed in triplicate.

$$\beta\text{-Galactosidase activity unit } (U/mL) = (OD_{420} \times A)/(B \times C \times 0.0045) \quad (1)$$

where A is the reaction mixture (mL), 5 mL; B is the reaction time (min), 40 min; C is the sample volume (mL), 10 mL; and 0.0045 (mL/n mol) is the extinction coefficient. Thus,

$$\beta\text{-Galactosidase activity unit } (U/mL) = OD_{420} \times 2.778 \quad (2)$$

2.6. Scanning electron microscopy

Logarithmic phase bacteria were allowed to adhere to poly-lysine-coated coverslips for 10 h and were exposed to cinnamaldehyde MIC for 6 h–8 h. The cells were washed in PBS after incubation and were fixed for 2 h at 4 °C with 2.5% glutaraldehyde. The cells were washed in the same buffer and were post-fixed for 30 min with osmium tetroxide in 0.1 M of cacodylate buffer (pH 7.2). The samples were dehydrated in ethanol, freeze-dried with a vacuum freeze drier (Hitachi ES-2030), coated with an ion sputtering apparatus (Hitachi E-1010), and observed through SEM (Hitachi S-3400N). The bacterial cells that were not exposed to cinnamaldehyde were similarly processed and used as control. All tests were performed in triplicate.

2.7. Transmission electron microscopy

Cells growing in beef-extract peptone medium were exposed to cinnamaldehyde MIC for 6 h–8 h. After incubation, the cells were harvested through centrifugation at 8000 r/min for 15 min at 4 °C, washed with PBS, and fixed for 24 h at 4 °C with 4% glutaraldehyde. The cells were washed in the same buffer and post-fixed for 2 h by using 1% osmium tetroxide in 0.1 M of cacodylate buffer (pH 7.2) at 4 °C. The samples were dehydrated in a graded acetone series and embedded in an embedding medium (Epon 812) for 3 h. The ultrathin sections were stained with uranyl acetate and lead citrate for 10 min and were observed through TEM (Hitachi H-7650). The bacterial cells not exposed to cinnamaldehyde were similarly processed and were used as the control. All tests were performed in triplicate.

2.8. Statistical analyses

All test series were performed in triplicate. All statistical tests were performed using the statistical program SPSS Version 11.5