Identification of bacillomycin D from *Bacillus subtilis* fmbJ and its inhibition effects against *Aspergillus flavus*

Qingwei Gong, Chong Zhang, Fengxia Lu, Haizhen Zhao, Xiaomei Bie, Zhaoxin Lu

College of Food Science and Technology, Nanjing Agricultural University, Weigang 1, Nanjing 210095, PR China

**A R T I C L E I N F O**

Article history:
Received 24 April 2013
Received in revised form 16 July 2013
Accepted 23 July 2013

Keywords:
Bacillomycin D
Aspergillus flavus
Antifungal
Food safety
Corn

**A B S T R A C T**

The study aimed to determine the structure and component of the antifungal substances against *Aspergillus flavus* produced by *Bacillus subtilis* fmbJ. With separation and purification, four of antifungal substances against *A. flavus* were obtained. By means of ESI-MS/CID analysis, their molecular weight was determined as being 1030D, 1044D, 1058D, 1072D, and the amino acid component of the antifungal sequence of bacillomycin D. The mechanism of bacillomycin D against *A. flavus* was investigated. Bacillomycin D exhibited a high antifungal effect on the mycelium growth, sporulation and spore germination of *A. flavus* with the inhibition rate of 85.72%, 98.10% and 96.63% respectively under 200 μg/mL of purified bacillomycin D. It was found that bacillomycin D could injure the cell wall and cell membrane of the hypha and spore, then, cytoplasms and organelles inside the cell were exuded and form empty hole by observation of SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy). The growth of *A. flavus* on corns could be completely inhibited in the presence of bacillomycin D at the concentration of 200 μg/g to 400 μg/g.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

*Aspergillus flavus* is weedy molds that grow on a large number of starch substrates. So it contaminates all major cereal crops with aflatoxins include cassava, chillies, corn, cotton seed, millet, peanuts, rice, sorghum, sunflower seeds, tree nuts, wheat, and a variety of spices intended for human or animal consumption. When processed, aflatoxins get into the general food supply where they have been found in human foods, as well as in feedstocks for agricultural animals. Aflatoxin transformation products are sometimes found in eggs, milk products and meat when animals are fed contaminated grains. The significant economic and health hazards caused by fungi and mycotoxin especially in developing countries that have poor food storages is of great concern so to ensure a healthy food supply thereby minimizing consequences to food security, international trade and animal and human health (Makun et al., 2010).

*A. flavus* is particularly common on corn and peanuts and is one of several species of mold known to produce aflatoxin (Mahmoudabadi, Zarrin, 2005; Tendolkar, Sharma, Mathur, Ranadive, & Sachdev, 2005). Aflatoxins are toxic metabolites produced by certain fungi in/on foods and feeds. They are still recognized as the most important mycotoxins. They, a group of extremely hazardous and common secondary metabolites, can cause acute hepatitis, immunosuppression and hepatocellular carcinoma (Hedayati, Pasqualotto, 2007), It’s one of the main reasons to induce to live cancer.

Therefore, in order to reduce risk of food safety due to *A. flavus* contamination, some studies about reduce or inhibit and, ultimately, eliminate *A. flavus* contamination are carrying out, which include directly using antagonistic microbes and extracting antifungal compounds from plants and microorganisms to inhibit *A. flavus* growth and spore germination (Norner, 2004). López-Malo, Alzamora, and Palou (2005) have investigated the combined effects of water activity (2aw] 0.99 or 0.95), pH (4.5 or 3.5) and antimicrobial agent (sodium benzoate, potassium sorbate, sodium bisulfite, carvacrol, citral, eugenol, thymol, or vanillin) concentration (0, 100, 200 up to 1800 ppm) on the growth of *A. flavus*, indicated that germination time of mold spore increased as antimicrobial agent concentration increased and when aw and pH decreased. The effects of some compounds from the plants such as citral and essential oil on *A. flavus* spores were studied (Luo & Jiang, 2004; Tian & Huang, 2012). There are few studies about bacterial metabolites against the growth of *A. flavus* and/or aflatoxin production. *Lactococcus lactis* subsp. Lactis CHD-28.3 (Roy, Batish, Grover, & Neelakantan, 1996), *Salmonella typhi* and *Escherichia coli* were found to secret the active compounds exhibited the antifungal
against *A. flavus* (Yadav et al., 2005). Obviously, we should find a method of controlling contamination of *A. flavus* and ensuring safety of food and feed.

The FDA stated that non-toxigenic and non-pathogenic strains of *Bacillus subtilis* are widely available and have been safely used in a variety of food applications. Some *B. subtilis* strains produce antimicrobial substances, such as lipopeptides, such as surfactin, fengycin and bacillomycin. Bacillomycin, a lipopeptide from *B. subtilis* was identified to have considerable antifungal against *A. flavus* (Moyne, Shelby, Cleveland, & Tuzun, 2001; Zhang, Shi, Hu, Cheng, & Wang, 2008). However, its antimicrobial mechanism and application have not been studied in detail. *B. subtilis* fmbJ (Liang & Lu, 2001) was isolated and identified by our laboratory, which has a broad spectrum of the antimicrobial activity. Recently, we found the fermentation broth from *B. subtilis* fmbJ has an inhibitory effect on *A. flavus*. So we tried, in the work, to identify the antifungal compounds against *A. flavus* and to elucidate the antifungal mechanism of the active compounds against *A. flavus*.

2. Materials and methods

2.1. Microorganism strain and culture conditions

*B. subtilis* fmbJ (CGMCCN 0943) is a wild-type strain originally isolated and characterized in our laboratory. *A. flavus* (CICCI2062), is provided by the Institute of Microbiology, Chinese Academy of Science, Beijing.

The strain fmbJ was inoculated in a 250 mL shake flask containing 100 mL of beef extract medium (3 g beef extract/L; 10 g peptone/L; 5 g NaCl/L; the pH was adjusted to 7.2) and cultivated at 37°C and 180 rpm/min for 24 h as a preculture. Concentration of the preculture reached 10^8–10^9 cfu/mL. Ten milliliters of preculture was inoculated in a 1000 mL shake flask containing 200 mL of Landy medium (20 g glucose/L; 1 g yeast extract/L; 5 g l-glutamic acid/L; 1.0 g KH2PO4/L; 0.16 mg CuSO4·5H2O/L; 0.5 g MgSO4·7H2O/L; 0.15 mg FeSO4·7H2O/L; 0.5 g/L KCl; 5.0 mg MnSO4/L, the pH was adjusted to 7.0 with 5 N NaOH), and then cultivated at 33°C at 180 rpm/min for 50 h for the antimicrobial substance production (Landy, Warren, Rosenman, & Colio, 1948).

2.2. Preparation of crude antifungal substances solution

The fermentation broth after centrifugation was treated with 6 M HCl. When its pH was adjusted to pH 2, then the precipitation (the supernatant has no activity against *A. flavus*) was obtained. The precipitation was readjusted with 4 M NaOH to pH 7, the methanol (100%) was used to extraction of the antifungal substances. After centrifuged at 10,000 g for 15 min, the solution of the crude antifungal substances was obtained.

2.3. Antifungal assay

Methods to determine antifungal activity are oxford cup: Potato dextrose agar (PDA) medium was used to antifungal experiments, which was prepared by dissolving 200 g potato, 20 g glucose and 15 g agar in 1000 mL distilled water. PDA plates containing 10^6 *A. flavus* spores per mL were prepared. Put the sterilized oxford cup in the center of the plate, then 200 µL of the antifungal substances from 2.2 was added into the Oxford cup, followed by aerobic incubation at 28°C for 48 h. The same volume of methanol (100%) was used as a control. The antifungal effect can be determined by observing the inhibition zone. The experiments were performed in triplicate.

2.4. High performance liquid chromatography (HPLC)

The sample was analyzed by reversed phase high performance liquid chromatography (C18 column, 250 mm × 4.6 mm, UltiMate 3000, USA). The mobile phase consisted of solvent A (HPLC grade water containing 0.1% trifluoroacetic acid (TFA)) and solvent B (HPLC grade acetonitrile with 0.1% TFA). A 20 µL aliquot of sample was loaded and separated on the column by an elution of linear biphasic gradient of 30–45% solvent B over 15 min, 45–55% over 40 min, at a flow rate of 0.6 mL/min. Elution was monitored by UV detection at 207 nm.

2.5. Separation and purification of the antifungal compounds

The crude compound solution was dissolved in 100% Methanol after evaporation. Then, the crude solution was applied to a Sephadex LH-20 column which had been equilibrated with methanol at a flow rate of 0.6 ml/min. Elution was performed with a discontinuous of methanol at 0.6 ml/min. Each fraction was tested for its antifungal activity against *A. flavus*. The active fraction was collected and concentrated for whole wavelength scanning and HPLC analysis.

Further purification was carried out by preparation HPLC with a C18 column on waters 600 and monitored by absorbance at 207 nm. The mobile phase consisted of solvent A (HPLC grade water containing 0.1% trifluoroacetic acid (TFA)) and solvent B (HPLC grade acetonitrile with 0.1% TFA) and the gradient elution program was the same as the RP-HPLC above.

2.6. LC–ESI mass spectrometry analysis of antimicrobial compounds

High performance liquid chromatography/electrospray ionization/collision-induced dissociation-mass spectrometry (HPLC/ESI/CID-MS) analysis was performed with Surveyor-LCQ DECA XP Plus of Thermo Finnigan (Thermo Electron Corporation, San Jose, CA, USA). The electrospray source was operated at a capillary voltage of 32 V, a spray voltage of 5 kV and a capillary temperature of 27°C.

2.7. Effects of bacillomycin D on hyphae of *A. flavus*

The inhibitory of hyphae growth was determined by growth rate assay. Bacillomycin D concentration of 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 ug/mL in each PDA (potato dextrose agar) plate were diluted by twofold dilution method, and the plate without bacillomycin D was regarded as contrast control. Hyphae discs (5 mm) of *A. flavus* were placed in the central of each PDA plate. The plates were incubated at 28°C. When the hyphae in the control plate reached the edges of the plate, the hyphae diameter of each plate with bacillomycin D was measured by decussation method. Growth inhibition of *A. flavus* was calculated as the percentage of inhibition of diametrical growth relative to the control.

The relative growth inhibition of treatment compared to control was calculated by percentage, using the following formula:

\[
\text{Inhibition} (\%) = \left(1 - \frac{\text{diametrical growth of treatment (mm)}}{\text{diametrical growth of control (mm)}}\right) \times 100\%
\]
All treatments were replicated 3 times and the test was conducted 2 times.

2.8. Effects of bacillomycin D on spore productivity of A. flavus

Hyphae discs (5 mm) of A. flavus were placed in the central of the PDA plate same to above. The plates were incubated at 28 °C for at least eight days. The spores was washed by 15 mL isotonic saline water (0.85% NaCl) containing 0.1% (v/v) Tween 20 and determined by a haemocytometer.

Inhibition (%) = \{ \text{the numbers of sporulation of the control} - \text{the numbers of sporulation of the experimental groups/the numbers of sporulation of the control} \} \times 100\%

There were 3 replicates for each treatment and the experiments were repeated twice.

2.9. Effects of bacillomycin D on spore germination of A. flavus

The Bacillomycin D powder were serially diluted with PDB (potato dextrose broth) to the final concentration of 400, 200, 100, 50, 25, 12.5, 6.25 μg/mL. Spore suspension was added to the PDB medium to the final concentration of 1.0 × 10⁶ cells/mL, and PDB medium with no spore was regarded as control. After incubation at 28 °C for 12 h in shaker at 120 r/min, the condition of spore germination was observed in optic microscope (Nikon E–100). When the length of germ tube was longer than the radius of spore, the spore was regarded to germinate. Germination inhibition of A. flavus was calculated as the percentage of inhibition of germination rate relative to the control. 10 microscopic filed were selected (10 × ) and at least 200 spores were observed. Each treatment was determined in triplicate and the experiments were repeated twice.

The relative germination inhibition of treatment compared to control was calculated by percentage, using the following formula:

Inhibition (%) = \{ 1 - \text{germination rate of treatment (%) / germination rate of control (%)} \} \times 100\%

All treatments were replicated 3 times and the test was conducted 2 times.

2.10. SEM (Scanning Electron Microscopy) and TEM (Transmission Electron Microscopy) observations on the hyphae and spores of A. flavus treated with bacillomycin D

The PDA plates with the bacillomycin D concentration of 100 μg/mL and control plates were obtained as described above. One hundred microliters of spor suspension of A. flavus (about 10⁶ conidia/mL) were spread uniformly on the surface of each plate. After incubation at 28 °C for 4 d, the hyphae grown on PDA were harvested for SEM, but for TEM, the hyphae were harvested after incubation at 28 °C for about 3 d. meanwhile, the spores for TEM were washed by isotonic saline water (0.85% NaCl) containing 0.1% (v/v) Tween 20 after incubation at 28 °C for about 8 d, then centrifuged at 3000 g for 3 min. The detailed method about SEM and TEM observations were described as below.

SEM: The fungal hyphae were fixed in 2.5% glutaraldehyde (prepared in 0.1 M sodium phosphate buffer) at 4 °C for 24 h, rinsed 3 times with phosphate buffer (0.02 M) and subsequently fixed with 2% osmium tetroxide for 2 h at 20 °C and dehydrated in a graded series of ethanol concentrations (30%, 50%, 75%, and 95%) for 10 min each, CO₂ dried and sputter coated with gold palladium in a Nanotech sputter coating apparatus (ES-2030 HITACHI, Japan). Samples were kept in a desiccator until examination with a scanning electron using a microscope (Philips, SEM-505, Holland) operated at 30 kV.

TEM: for TEM, the fungal hyphae and spores used the similar procedure (SEM) until dehydrated. After dehydration, the samples were embedded in Epon810 and the ultra thin section (80 nm) at room temperature was cut on an ultra-microtome RMC-MT7000 and diamond knife. Once the tissue had been mounted on a copper grid, a post staining was carried out (uranyl acetate for 30 min and lead citrate for 20 min). Samples were kept in the desiccators until examination with a TEM (HITACHI, H-600, Japan) operated at 120 kV.

2.11. Antifungal activity of purified bacillomycin D on corns

The purified bacillomycin D was weighed, and then was dissolved in sterile distilled water. Corns were surface sterilized with 1% NaOCl solution and rinsed in sterile distilled water for at least three times. Ten grams of corns were distributed in each of 100 mL sterilized triangular flasks and 2 mL of each concentration of bacillomycin D was added to each flask. For the control, 2 mL sterile distilled water was replaced. The flasks were kept at 180 rpm/min for 24 h so that all the solution could be absorbed by the corns.

Then, 150 μL spore suspensions of A. flavus (about 10⁶ conidia/mL) were incubated in each flask. After incubation at 28 °C for about 7 days, the growth of A. flavus was evaluated macroscopically.

2.12. Statistical analysis

To test the significance of the treatment effect, the data from all experiments conducted in this study were analyzed using Duncan’s multiple range test (p < 0.05), following one-way ANOVA. The statistical analysis was performed using statistical software (SPSS, 15.0).

3. Results

3.1. Purification and identification of the antifungal compounds

By HCl precipitate from the fermentation broth and subsequent Sephadex LH-20 chromatography, the antimicrobial substances
were separated (Fig. 1). In order to confirm the active peak, further purification was necessary for the active fractions. With reparative reversed phase chromatography as well antifungal assay, four peaks with antifungal activity were purified (Fig. 2a). Therefore, these four purified peaks were subjected to their structural identification.

The molecular weight of the purified chemicals having antifungal activity were elucidated by LC-ESI mass spectrometry. Data from the positive ion mass spectrum analysis (Fig. 2b) showed that [M+H]+ ion peaks were 1031.8 (purified peak 1), 1045.9 (purified peak 2), 1059.7 (purified peak 3), and 1073.9 (purified peak 4), respectively. So, the molecular weight of four activity peaks were 1030 Da (peak 1), 1044 Da (peak 2), 1058 Da (peak 4), and 1072 Da (peak 6).

The antifungal peptides secreted by *B. subtilis* have a molecular weight of less than 2000 Da and belong to cyclic lipopeptides. Intervals of 14 are often observed for molecular weight of these cyclic lipopeptide with different numbers of methylene groups (—CH2—) in fatty acyl-chains. By comparing with the mass data with those obtained in previous studies, the purified peaks with antifungal activity against *A. flavus* may be identical to bacillomycin D (Peypoux et al., 1984; Moyne et al., 2001).

ESI/CID-MS spectrometry analysis of [M+H]+ (m/z 1045.9) was carried out in order to further confirm whether being bacillomycin D. Fig. 3 showed ESI/CID-MS spectrum of precursor ions (m/z 1045.36) and elucidated the composition of the amino acid or peptides. It was found that the composition of amino acid residue was {Asn-Tyr-Asn-Pro-Glu-Ser-Thr}, which agreed with the amino acid composition of Bacillomycin D (Moyne, Cleveland, & Tuzun, 2004). Summarized above analysis, the active peaks could be identified as the homologues of bacillomycin D with C14 to C17 fatty acid chain.

3.2. Effects of bacillomycin D on hypha growth in *A. flavus*

Bacillomycin D could obviously inhibit the hyphae of *A. flavus* (Table 1). When the bacillomycin D concentration was 50 μg/mL, the inhibition rate to hyphae reached to 27.52%. The higher the bacillomycin D concentration, the more the inhibition rate to hyphae growth was. When the bacillomycin D concentration was set at 200 μg/mL, the inhibition of hypha growth was found to be 85.72%.

3.3. Effects of bacillomycin D on sporulation and spore germination of *A. flavus*

Table 2 showed that bacillomycin D significantly inhibited the formation of the spores of *A. flavus*. The spore numbers of *A. flavus* formed on plates gradually reduced and the inhibition rate increases with increase of the concentration of bacillomycin D. When the bacillomycin D concentration was 200 μg/mL, the inhibition rate was found to be 98.10%.

The germination of fungal spore was divided into three phases, the expansion of spore ball, the emergence and growth of germ tube as well the formation of hypha. *A. flavus* spores were incubated in the PDB (potato dextrose broth) medium containing different concentration of bacillomycin D. After incubation of 12 h, most spores without bacillomycin D treatment (the control) germinated, the spore balls expanded and the germ tube had been formed. However, the germination of spores with bacillomycin D treatment decreased gradually (Table 2). When the bacillomycin D concentration was 200 μg/mL, the inhibition rate was found to be 96.63%. Moreover, in the experimental group, for the germinated spores,
the spore balls got small, the length of germ tube got short, and some spore balls even were distorted. It was obvious that the bacillomycin concentration for completed inhibiting spore former and spore generation was about 200–400 μg/mL.

3.4. SEM and TEM observations on the hyphae and spores of A. flavus treated with bacillomycin D

The surface appearance of hyphae and spores was observed by SEM. The non-treated hyphae grow normally, were smooth with no breakage (Fig. 4A) while the structures of bacillomycin D-treated hyphae were damaged and changed irregular (Fig. 4B). Treated with bacillomycin D, the hyphae were deformed and distorted. There were introcession, enation, wilting on the surface of hyphae; the edge was blurry while hyphae seemed cavitate (Fig. 4A, B). SEM observation for the spores also indicated there was obviously change in the spore morphology of A. flavus, the spores without bacillomycin D grow normally (Fig. 4C). However, the bacillomycin D intensely induced the abnormality and deformation of Spore stalk, sporangium and spores (Fig. 4D). The Spore stalk and sporangium were twisted, distorted and shrinking. Spores were deformed, and could not grow normally.

The internal structure of hyphae and spores was observed by TEM. TEM observation could further elucidate morphological changes of hyphae and spores of A. flavus induced by bacillomycin D. When treated without bacillomycin D, the structure of hyphae cell were intact, the mycelial cell all is clear, complete with smooth edge and all cell component especially the cellular organ were visible arranged in order, apparently (Fig. 4E). Nevertheless, almost all the cytoderm of the hyphae treated with bacillomycin D were dissolved, and became thin, fuzzy. The cell wall was lumpy. Cell membrane and cytoplasm were uneven as well. Furthermore, the organelles and cytoplasms were irregular and degenerated even appeared some empty holes (Fig. 4F).

With regard to the spore, the untreated spore cell remained intact and all cell components especially the cellular organ were arranged in order. Besides, the surface of the spores was relatively smooth (Fig. 4G). But spores were seriously deformed and distorted of the treated spores. The cell wall was lumpy and the cytoderm of some spores were damaged with larger cell gap, there was also large space of vacuity inside the cell (Fig. 4H). The results of SEM and TEM suggested that bacillomycin D could injure the cell wall of

Table 1
The effect of Bacillomycin D on the inhibition of hyphae growth in A. flavus.

<table>
<thead>
<tr>
<th>Bacillomycin D concentration (μg/mL)</th>
<th>Colony diameter (mm)</th>
<th>Inhibition(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>79.68 ± 0.45 a</td>
<td>0 a</td>
</tr>
<tr>
<td>1.56</td>
<td>78.92 ± 0.23 a</td>
<td>0.95 b</td>
</tr>
<tr>
<td>3.13</td>
<td>78.32 ± 0.11 c</td>
<td>1.71 c</td>
</tr>
<tr>
<td>6.25</td>
<td>76.57 ± 0.12 d</td>
<td>3.90 d</td>
</tr>
<tr>
<td>12.5</td>
<td>74.65 ± 0.18 e</td>
<td>6.31 e</td>
</tr>
<tr>
<td>25</td>
<td>72.67 ± 0.46 f</td>
<td>8.00 f</td>
</tr>
<tr>
<td>50</td>
<td>57.75 ± 0.61 g</td>
<td>27.52 g</td>
</tr>
<tr>
<td>100</td>
<td>27.83 ± 0.37 h</td>
<td>65.07 h</td>
</tr>
<tr>
<td>200</td>
<td>11.38 ± 0.17 i</td>
<td>85.72 i</td>
</tr>
<tr>
<td>400</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Inhibition values were calculated using the formula: Inhibition (%) = [1 – diametrical growth of treatment (mm)/diametrical growth of control (mm)] × 100. Data were pooled from 2 trials. Values in each plate followed by different letters are statistically different by Duncan’s multiple range test (P < 0.05).

Table 2
The influence of Bacillomycin D on sporulation of Aspergillus flavus.

<table>
<thead>
<tr>
<th>Bacillomycin D (μg/mL)</th>
<th>Sporulation × 10^5</th>
<th>Inhibition (%)</th>
<th>Generation (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>280 ± 3 a</td>
<td>0 a</td>
<td>99.00 ± 2.31 a</td>
<td>0 a</td>
</tr>
<tr>
<td>6.25</td>
<td>242 ± 3 b</td>
<td>13.57 b</td>
<td>93.00 ± 1.00 b</td>
<td>6.06 b</td>
</tr>
<tr>
<td>12.5</td>
<td>228 ± 1 c</td>
<td>18.45 c</td>
<td>83.00 ± 2.46 c</td>
<td>16.16 c</td>
</tr>
<tr>
<td>25</td>
<td>211 ± 3 d</td>
<td>24.76 d</td>
<td>72.00 ± 1.65 d</td>
<td>27.27 d</td>
</tr>
<tr>
<td>50</td>
<td>109 ± 2 e</td>
<td>61.19 e</td>
<td>56.67 ± 2.52 e</td>
<td>43.77 e</td>
</tr>
<tr>
<td>100</td>
<td>35 ± 1 f</td>
<td>87.62 f</td>
<td>39.67 ± 2.08 f</td>
<td>70.03 f</td>
</tr>
<tr>
<td>200</td>
<td>5 ± 1 g</td>
<td>98.10 g</td>
<td>3.33 ± 1.53 g</td>
<td>96.63 g</td>
</tr>
<tr>
<td>400</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Values in each plate followed by different letters are statistically different by Duncan’s multiple range test (P < 0.05).
the hypha and spore and cross the cell membrane. Then, cytoplasm and organelles inside the cells were damaged to form empty hole and exude. We can also conclude that the effects of the same concentration of bacillomycin D on the hyphae of A. flavus were stronger than that of the spores. It could be attributed to the thicker cell walls of spores.

3.5. Antifungal activity of bacillomycin D on corns

After incubation for 8 days, the contamination of A. flavus on corns was examined in the presence of purified bacillomycin D. The results indicated that the growth of A. flavus decreased with the increased concentration of bacillomycin D. Compared one without bacillomycin D, 50 μg/g and 100 μg/g of bacillomycin D could significantly reduce A. flavus growth, and at the concentration of 200 μg/g to 400 μg/g, there was no A. flavus observed, so the growth of A. flavus can be completely inhibited (Table 3).

4. Discussions

The three families of lipopeptides, such as surfactins, iturins and fengycins are amphiphatic molecule and synthesized non-ribosomally via large multi-enzymes. They have been well-recognized potential uses in medicine, agriculture and the food industry because of their surfactant properties (Ongena & Jacques, 2008). Members of the fengycins and surfactins families are composed of one hydroxy fatty acid and ten (fengycins) or seven (surfactins) amino acids. Iturins are composed of one amino fatty acid and seven amino acids. Bacillomycin D belongs to the iturins, indicating that bacillomycin injured cell walls of hyphae and spores.

Different letters for the same measurement show significance (P < 0.05). Data expressed as means ± SD, n = 3.

Table 3
The results of bacillomycin D inhibit A. flavus on corns at different concentration.

<table>
<thead>
<tr>
<th>Bacillomycin D concentration (μg/g)</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>300</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>The growth of A. flavus(%)</td>
<td>100 ± 0 a</td>
<td>88 ± 1 b</td>
<td>35 ± 2 c</td>
<td>9 ± 2 d</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>0</td>
<td>12</td>
<td>65</td>
<td>91</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

The results of bacillomycin D inhibit A. flavus from B. Subtilis AU195 (Moyne et al., 2001) and Zhang et al. (2008) purified bacillomycin D-like (masses of 1058 and 1072 Da) compounds inhibiting the growth of A. flavus from B. Subtilis B-FS06. All of the other studies about bacillomycin D had nothing to do with A. flavus (Tabbene et al., 2011; Koumoutsi, Chen, 2007; Oleinikovaet et al., 2005). In this studies, by the means of acid precipitation, Sephadex LH-20 column chromatography, preparative high performance liquid chromatography and ESI/CID-MS analysis, the antifungal substances were purified and identified as four homologous compounds of bacillomycin D (masses of 1030, 1044, 1058 and 1072 Da) with the fatty acyl chain C14 to C17, indicated that B. subtilis fmbj produced more kinds of bacillomycin than that previously reported.

Romero, Vicente, Olmos, Davila, and Perez-Garcia (2007) analysed the morphological and ultrastructural effects of lipopeptides of cell-free liquid cultures from the antagonistic B. subtilis strains on the cucurbit powdery mildew fungus, Podosphaera fusca, showed that the lipopeptides reduced cucurbit powdery mildew disease by arresting conidial germination, which resulted from the induction of important cytological alterations. In this work, we first studied the antifungal effects of purified bacillomycin D from the morphology and ultra-structure of A. flavus. We have demonstrated that bacillomycin D can significantly inhibit mycelial growth, spore productivity, and spore germination in A. flavus. SEM and TEM observations of hyphae and spores of A. flavus in the presence of bacillomycin D showed severe ultra-structural alterations of them, indicating that bacillomycin injured cell walls of hyphae and spores and crosses cell membranes. Cytoplasm and organelles were damaged to form empty holes and exudates. Many reports have shown that lipopeptides such as surfactin, iturin, and fengycin can damage cell membranes; these are the prime sites for antimicrobial attack by surfactin (Deleu et al., 2003; Carrillo, Teruel, Aranda, & Ortiz, 2007), iturin (Régine, Peypoux 1994), and fengycin (Deleu, Paquot, & Nylander, 2008; Tao, Bei, Lv, Lu, 2011). Although bacillomycin D can damage cell membranes, we conclude that the prime site for bacillomycin D attack could be cell walls, following our TEM observations that the cell walls of hyphae were damaged and the cell walls of spores seriously deformed, at the same concentrations of bacillomycin D.

There were more studies on bacillomycin, but the research on controlling the growth of A. flavus using bacillomycin in vivo was...
few reported. Zhang et al. (2008) have also found that bacillomycin-like compounds could control the contamination of A. flavus on peanuts. In the work, we investigated the inhibiting effect of purified bacillomycin D on the growth of A. flavus on corn, and have found the bacillomycin D could effectively inhibit the growth of A. flavus on corn, indicating that bacillomycin D may be a potential preservative for food/feed storage.

A. flavus grow easily on the all major cereal crops and secret aflatoxins, which severely affect human health and food safety, therefore a large of food/feed loses happen with aflatoxins contamination in the global. As a lipopeptide derived from B. subtilis, the bacillomycin D has effective antifungal activities and safer for humans and the environment and does not have the negative impact of antibiotic resistance. Therefore, the bacillomycin D may have a broad application in biological control and food/feed preservation, and enable reduction in the pollution of food and feed. Finally, the application of bacillomycin will save a large of food/feed loses with aflatoxins contamination and guarantee food safety.

Acknowledgment

This work was supported by grants from the National Natural Science Foundation of China (No. 31271936) and the National Research Program of China (No. 2011BAD23B05).

References


