World J Microbiol Biotechnol (2008) 24:783–788
DOI 10.1007/s11274-007-9533-1

ORIGINAL PAPER

Antifungal compounds from *Bacillus subtilis* B-FS06 inhibiting the growth of *Aspergillus flavus*

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Received: 12 March 2007 / Accepted: 8 August 2007 / Published online: 2 September 2007

Abstract The cell-free culture filtrate (CCF) was prepared from a culture of an *Aspergillus flavus* antagonist, *Bacillus subtilis* B-FS06. The CCF inhibited the growth and spore germination of *A. flavus* at a series of concentrations (10, 25, 50%) (v/v). It still retained the activity after treatment at pH values ranging from 2 to 12 for 24 h or at 100 °C for 30 min. The antifungal activity, however, was reduced by 30% after treatment at 121 °C for 20 min. After purification by anion exchange chromatography, gel filtration chromatography and HPLC, the active compounds revealed six ion peaks: [M–H] m/z = 1006.78, 1020.71, 1034.74, 1049.54, 1056.78, and 1071.64 by using electrospray ionization mass spectrometry (ESI-MS) analysis. In the presence of the active compounds at 200 μg/g, the growth of *A. flavus* on peanuts was completely inhibited.

Keywords *Aspergillus flavus* · *Bacillus subtilis* · Bacillomycin D · Surfactin

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Introduction

Aspergillus flavus is one of the major spoilage organisms of intermediate moisture foods (Nielsen and Rios 2000; Batista et al. 2003; González et al. 2005). It is notable for the secondary metabolites (aflatoxins) that are potent hepatotoxins and carcinogens (Cullen and Newberne 1994; Roebuck and Maxuitenko 1994). Aflatoxins were designated as human liver carcinogens in 1993. Besides producing aflatoxins, *A. flavus* is also a human pathogen causing aspergillosis and onychomycosis (Tendolkar et al. 2005; Zhang et al. 2005; Mahmoudabadi and Zarrin 2005). Of the many research approaches being used to reduce and, ultimately, eliminate *A. flavus* contamination, biological control is one of the more promising techniques, particularly for the near-term (Norner 2004).

Numerous bacteria have been tested for biological control of *A. flavus*. Cuero et al. (1987) investigated the interaction between *A. flavus* and *Hyphopichia burtonii* or *Bacillus amyloliquefaciens* in the laboratory, which indicated that temperature and water activity played key roles in determining whether these two bacteria act as inhibitors or stimulators on the growth and aflatoxin production of *A. flavus*. Kimura and Hirano (1988) isolated a *B. subtilis* strain NK-330 that could inhibit the growth and aflatoxin production of *A. flavus*. Misaghi et al. (1995) screened a *Pseudomonas cepacia* (D1) strain from 892 bacterial isolates, which suggested that the bacterium significantly reduced the damage of *A. flavus* to cotton locules when D1 was inoculated with *A. flavus* simultaneously in field studies. Bueno et al. (2006) determined the effects of two species of lactobacilli, *Lactobacillus casei* CRL 431 and *L. rhamnosus* CRL 1224, on the growth of different *A. flavus* strains. The results indicated that *L. casei* CRL 431 and *L. rhamnosus* CRL 1224 might be utilized as potential biocontrol agents against *A. flavus*. Some other bacterial species had been found to be able to reduce *A. flavus* growth and/or aflatoxin production significantly (Mickler et al. 1995; Misaghi et al. 1995; Bluma and Etcheverry 2006; Palumbo et al. 2006).

It is not feasible by using bacteria for direct biological control under any conditions. In this case, biorational control, the direct use of active compounds rather than bacteria, is an attractive alternative control strategy (Klich et al. 1993). However, few studies have been performed on the identification of inhibitory bacterial metabolites against the growth of *A. flavus* and/or aflatoxin production. The cell-free supernatant of *Lactococcus lactis* subsp. *lactis* CHD-28.3 (Roy et al. 1996) and the culture supernatant and lysate of *Salmonella typhi* and *Escherichia coli* (Yadav et al. 2005) exhibited the activities against *A. flavus*. The active compounds, however, have not yet been purified and/or identified. Only bacillomycin D, a lipopeptide from *Bacillus subtilis* AU195, was found to inhibit the growth of *A. flavus* (Moyne et al. 2001). During the screening of bacteria for antagonistic activity against *A. flavus* in vitro, we identified a *Bacillus subtilis* isolate, B-FS06 with high antifungal activity (Zhang et al. 2007).

The purpose of this study was to identify the influence of the cell-free culture filtrate (CCF) on the mycelial mass and spore germination of *A. flavus*, and to isolate, purify and characterize the antifungal compound(s) secreted by *B. subtilis* B-FS06.

Materials and methods

Microorganisms

Bacterial antagonist, strain B-FS06, was isolated from a rape field and identified as *Bacillus subtilis* (Zhang et al. 2007). The strain was stored on nutrient agar (NA) (1% peptone, 0.3% beef extract, 0.5% NaCl, 1.5% agar, pH 7.0) slants at 4 °C. *A. flavus* CGMCC 3.2890 was provided by the China General Microbiological Culture Collection Center (CGMCC). Potato dextrose agar (PDA) medium (20% potato extract, 2% dextrose, 1.5% agar) slants were used to store the fungus at 4 °C.

Preparation of CCF

The B-FS06 strain was activated in 3 mL nutrient broth (NB) medium (1% peptone, 0.3% beef extract, 0.5% NaCl, pH 7.0) on a constant temperature shaker (30 °C, 120 rev/min) for 8 h. The broth was transferred to 2,000 mL fresh NB and incubated at 30 °C, 120 rev/min for 48 h and centrifuged at 1,000*g* for 20 min at 4 °C. The supernatant was concentrated to five times of the original concentration by using polyethylene glycol 6000 and filtered through a 0.45 μm pore size filter to yield the CCF. It was stored at –20 °C.

In vitro antifungal activity

Agar well diffusion assay was used for the detection of antifungal activity. PDA plates containing 10^4 *A. flavus* spores per mL were prepared. A well with a diameter of 6 mm was then cut in the agar using a sterile cork-borer. A droplet of agar was added to the well in order to seal it to avoid leakage. Then, 100 μL of CCF was added into the well and allowed to diffuse into the agar during a 5-h pre-incubation period at room temperature, followed by aerobic incubation at 30 °C for 24 h. The antifungal zone was recorded.

Inhibition of fungal mycelium by CCF

CCF was added to autoclaved and pre-cooled potato dextrose broth (PDB) in 100-mL flasks at concentrations of 50, 25, 10% (v/v) to a final volume of 30 mL. The control flask was used without CCF. Each treatment flask was inoculated separately in triplicate with 10 μL of *A. flavus* spores suspension containing 1×10^5 spores. Flasks were incubated at 30 °C in a shaking incubator at 150 rev/min. Mycelium was harvested after 5 days, freeze-dried, and the mycelial weight was recorded.

Spore germination inhibition assay

The inhibitory effect of CCF against spore germination of *A. flavus* was tested in microtiter plates. Each well contained 90 μL mixture (PDB mixed with the CCF and sterile water), and then 10 μL spore suspension containing 1×10^4 spores was added into the well. The final concentrations (v/v) of CCF were 0, 10, 25, and 50% respectively. The slides were placed in humid chambers in triplicate and incubated in the dark at 30 °C for 8 h. Immediately after incubation, a drop of lactophenol cotton blue (40 mL of glycerol, 20 mL of lactic acid, 20 g of phenol, and 5 mL of 1% aqueous cotton blue) was added to each slide to prevent further growth and then the number of germinated and non-germinated spores was counted under a light microscope (Nikon YS100, Tokyo, Japan). In each replication, 100 spores were observed, and the percentage of spore germination was then calculated.

Stability of CCF

In order to test the stability of CCF at different temperatures, the samples of CCF were exposed at 20, 40, 60, 80, 100 °C for 30 min or 121 °C for 20 min. The remaining antifungal activities were assayed after the solutions had cooled to room temperature. In order to test the stability of CCF under acid and alkaline conditions, CCF samples were adjusted to pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12.0 using 0.5 M HCl or NaOH, and maintained at room temperature for 24 h. After the pH had been readjusted to 7.0, the remaining anti-*Aspergillus* activities were determined. The antifungal activities were tested by using the agar well diffusion assay.

Isolation of the antifungal compound

The pH of the CCF was adjusted to 2 with concentrated HCl. After centrifugation at 1,300*g* for 20 min at 4 °C, the precipitate was extracted twice with five times volume of methanol. The crude extract was dissolved in 50 mM Tris–HCl, pH 7.5 buffer after evaporation of the methanol. The solution was loaded onto a Cellulose DE52 (1.6 × 15 cm) column equilibrated with 50 mM Tris–HCl, pH 7.5 buffer which containing 50 mM NaCl. The column was washed with 150 mL of the same buffer and then with 150 mL of a linear 0.05–1 M NaCl gradient at a flow rate of 1 mL/min. The outflow was monitored by absorbance at 280 nm. The antifungal fraction was then applied to a Sephadex G-100 (1.5 × 80 cm) column which had been equilibrated with distilled water and eluted with distilled water at a flow rate of 0.8 mL/min. The antifungal fraction was collected and concentrated. Further purification was carried out by RP-HPLC with a C18 column (COSMOSIL 5C18-AR-300, 4.6 × 250 mm, Waters) on Agilent 1100 and monitored by absorbance at 215 nm. Elution (0.8 mL/min) was performed with 40% methanol in water during 0–10 min, a linear gradient from 40% methanol in water to 95% during 10–40 min. 95% methanol in water during 40–50 min. The major peaks were collected manually and subsequently tested for antagonistic activity against *A. flavus*.

Identification by mass spectrometry

Electrospray ionization mass spectrometry (ESI-MS) analysis was performed on a Finnigan TSQ Quantum ultra triple quadrupole mass spectrometer (Thermo Electron Corporation, San Jose, California, USA) to determine the molecular weight of the antifungal compounds. The TSQ Quantum Ultra was operated with negative ionization mode, electrospray voltage set at 5,000 V and ion transfer tube temperature at 350 °C.

Antifungal activity of the compounds on peanuts

The compounds were freeze-dried and weighed, and then were dissolved in 50 mM Tris–HCl, pH 7.5. Peanuts were surface sterilized with 1% NaOCl solution and rinsed in three successive changes of sterile distilled water. Fifteen grams of peanuts were distributed in each of 100 mL conical flasks and 2 mL of each concentration of compounds was added to each flask. For the control, 2 mL 50 mM Tris–HCl (pH 7.5) was added to 15 g peanuts in conical flasks. The flasks were kept for 24 h with thorough agitation so that all the solution could be absorbed by peanuts. The flasks were then inoculated in triplicate for each concentration with 100 μL of *A. flavus* spore suspension (1×10^4). After incubation at 30 °C for 5 days with vigorous shaking for 5 min daily, the growth of *A. flavus* was evaluated macroscopically.

Results

Effect of CCF on mycelial weight and spore germination

The experiment of the mycelial weight inhibition by CCF was determined after 120 h of incubation. Mycelia were freeze-dried and weighed. Compared with black control, there was a significant reduction in the weight of fungal mycelia in the presence of CCF, which reduced by more than 50% when the concentration of CCF attained 25%.

In order to determine the inhibitory effect of CCF on spore germination, *A. flavus* spores were inoculated in microtiter plates in the present of CCF. After incubation for 8 h, the percentage of spore germination was calculated. Our data suggested that CCF also inhibited spore germination. Compared with control (without CCF), spore germination rate reduced by more than 40% by 25% of CCF.

Effect of temperature and pH on CCF

After exposure at different temperatures and pH values, the CCF was stable enough for routine laboratory studies. No activity loss was observed after the incubation of CCF at 20–100 °C for 30 min. However, the activity reduced by 30% when the CCF was autoclaved at 121 °C for 20 min. The activity of CCF was not affected at pH 2 through 12.0 after incubation at room temperature for 24 h.

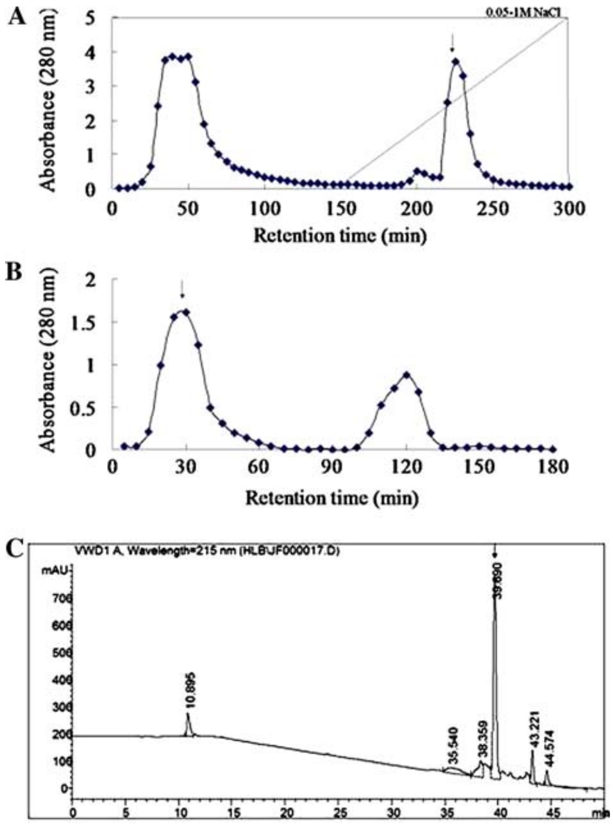

Fig. 1 Purification of the active compounds. (**A**) Anion-exchange chromatography on cellulose DE52 column. Column was eluted with Tris–HCl buffer at a flow rate of 1 mL/min, detected at 280 nm. (**B**) Gel filtration chromatography on Sephadex G-100 column. The column was eluted with distilled water at a flow rate of 0.8 mL/min, detected at 280 nm. (**C**) RP-HPLC with C18 column. Mobile phase: water, methanol. Flow rate: 0.8 mL/min. Detector: 215 nm. The arrowhead indicated the active fraction

Purification of the active compounds

The HCl precipitate of the *B. subtilis* B-FS06 CCF was extracted with methanol. After removal of methanol, crude extract was dissolved in 50 mM Tris–HCl buffer, pH 7.5 and passed through a cellulose DE52 anion-exchange column (Fig. 1A). All the peaks were collected as separate fractions, and the peak eluting at 220 through 250 min showed antifungal activity. The fraction with anti-*Aspergillus* activity was then purified on a Sephadex G-100 gel filtration column (Fig. 1B). Two peaks were obtained and the first peak showed the antifungal activity. RP-HPLC was performed for further purification of active compounds (Fig. 1C). The major peak (40 min) collected manually showed strong antifungal activity.

Mass spectrometry analysis

Mass spectrometry was utilized to identify the antifungal compounds. The mass spectrum (Fig. 2) revealed six ion peaks: [M–H] *m/z* = 1006.78, 1020.71, 1034.74, 1049.54, 1056.78 and 1071.64. The peak [M–H] *m/z* = 1071.64 had the highest relative abundance.

Inhibitory activity in vivo

The contamination of *A. flavus* on peanuts was examined after incubation for 5 days in the presence of active compounds (Fig. 3). The growth of *A. flavus* decreased with the increase of the active compounds concentration. Compared with the blank control, 100 μg/g of active compounds significantly reduced mycelial growth, and the growth of *A. flavus* was completely inhibited at 200 and 250 μg/g of compounds.

Discussion

To search for antifungal compounds with high activity against *A. flavus*, *B. subtilis* B-FS06 was isolated with antagonistic activity against *A. flavus* (Zhang et al. 2007). The CCF of *B. subtilis* B-FS06 significantly inhibited the growth and spore germination of *A. flavus*, and its antifungal compound is very heat stable and insensitive to pH. Of the two propagules, *A. flavus* spores are most ubiquitous as agents of reproduction, dispersal and/or survival, and hence have the largest dispersal scope and potential of infecting plant species and contaminating food and feed. Since germination is the starting event of the asexual life cycle of this fungus, antifungal compounds from *B. subtilis* B-FS06 should feasibly prevent germination of this fungus. Furthermore, purified compounds could inhibit the growth of *A. flavus* on peanuts, which indicated that the compounds had the potential as additive applied in food storage.

Chromatographic analysis of the HCl precipitate from B-FS06 showed that the antifungal compounds were proteins. Furthermore, mass spectrum analysis revealed six peaks with *m/z* between 1000 and 1100. Most of the antifungal peptides secreted by *B. subtilis* have a molecular weight of less than 2,000 Da and belong to cyclic lipopeptides with a peptide moiety and a fatty acid linked to the constituent amino acid residues. Intervals of 14 are often observed for molecular weight of these cyclic lipopeptides with different numbers of methylene groups ($-CH_2-$) in fatty acylchains. These antifungal compounds were separated from two series of ion peaks: [M–H] *m/z* = 1006.78, 1020.71, 1034.74, 1049.54 and [M–H] *m/z* = 1056.78, 1071.64 according to their *m/z*. The two series of peaks were highly similar to surfactin and bacillomycin D homologues respectively, by comparing with their mass data with those obtained in previous studies (Kowall et al. 1998;

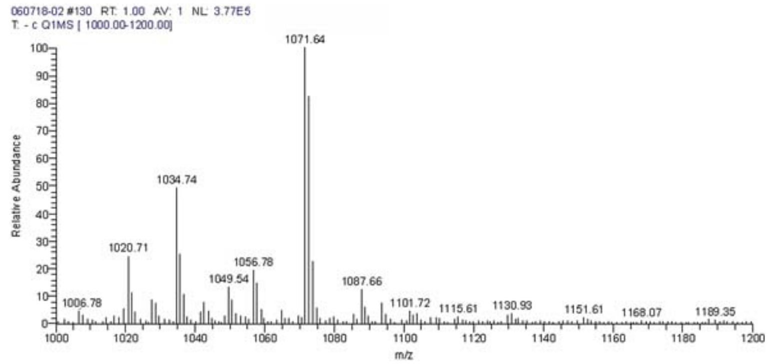

Fig. 2 Mass spectrum of the antifungal compounds on Finnigan TSQ Quantum ultra triple quadrupole mass spectrometer. The TSQ Quantum Ultra was operated with negative ionization mode, electrospray voltage set at 5,000 V and ion transfer tube temperature at 350 °C

Fig. 3 Purified compounds inhibit *A. flavus* on peanuts at a series of concentration: (**A**) blank control; (**B**) 50 μg/g; (**C**) 100 μg/g; (**D**) 150 μg/g; (**E**) 200 μg/g; (**F**) 250 μg/g

Koumoutsi et al. 2004; Moyne et al. 2001). Surfactin and bacillomycin D are lipopeptide biosurfactants produced by *B. subtilis*. Surfactin has hemolytic (Kracht et al. 1999), antiviral (Kracht et al. 1999; Vollenbroich et al. 1997), antibacterial (Heerklotz and Seelig 2001) and antitumor (Kameda 1974) properties, while bacillomycin D has antitumor (Oleinikova et al. 2005), hemolytic (Oleinikova et al. 2005) and antifungal (Moyne et al. 2001) activities. Compared with previous studies, we suggest that the bacillomycin-like compounds are responsible for the anti-*Aspergillus* activity.

Bacillomycin D belongs to the iturin group. Iturin is a group of cyclic lipopeptides including iturin A, C, D, bacillomycin D, F, L and mycosubtilin (Maget-Dana and Peypoux 1994). Ono and Kimura (1991) reported that iturin A could inhibit aflatoxin production by *A. flavus*, but later iturin A was described not to be able to inhibit *A. flavus* growth and aflatoxin production (Klich et al. 1993; Moyne et al. 2001). Moyne et al. (2001) purified two bacillomycin D analogues (masses of 1,044 and 1,058 Da) with inhibitory activity of *A. flavus* from *B. subtilis* AU195. In this paper, we purified bacillomycin-like compounds with masses of 1,058 and 1,072 Da, which indicated one methylene group (–CH_2–) in the fatty acyl chain more than that which Moyne et al. (2001) described. The biological activity of iturin depends on the composition of the length of the lipid chain. The longer the lipid chain length, the greater the antifungal activity of iturin (Maget-Dana and Peypoux 1994). This suggests that B-FS06 has a stronger antifungal activity than *B. subtilis* AU195.

Conclusion

In conclusion, bacillomycin-like compounds were purified and identified according to its anti-*Aspergillus* activity. *A. flavus* occurs widely during intermediate moisture food storage. Because of their activity against *A. flavus*, the compounds may be useful as potential biocontrol agents against *A. flavus* during food storage.

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