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Antifungal Effects of Lipopeptide Produced by *Bacillus amyloliquefaciens* BH072

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Abstract

An antifungal lipopeptide iturin A with strong activity against *Fusarium oxysporum* was produced by honey isolated strain *Bacillus amyloliquefaciens* BH072. For large-scale biocontrol application, the antifungal effect was deeply demonstrated by structure and mode of action. Cyclic structure and second structure were determined based on situ acid hydrolysis and Fourier Transformed-Infra Red (FT-IR) Spectra analysis. Structure of α-helix was predicted which might be associated with activity. Afterwards, antifungal mechanism of iturin A on *F. oxysporum* were investigated from fungal cell wall to the plasma membrane and finally to intracellular proteins by morphological, activity of alkaline phosphatase (AKP), conductivity, Malondialdehyde (MDA) and SDS-PAGE detection. Antifungal damage appears on not only the spore germination and mycelium growth of *F. oxysporum*, but also the leakage of cellular proteins. Moreover, growth of *F. oxysporum* could be inhibited in the presence of iturin A at a MIC of 2.5 mg/mL. Considered with its high production in previous work, *B. amyloliquefaciens* strain BH072 and iturin A might be a promising candidate for biocontrol.

Keywords

Antifungal Effect, Iturin A, *Bacillus amyloliquefaciens*, *Fusarium oxysporum*

1. Introduction

Deep fungus infections are widespread in nature. Fungal contamination causes not only huge economic losses, but also food safety issues. Fungi also lead to industrial contamination and some result in human and animal diseases [1]. Prevention and control of fungal contamination is an important issue in the field of

industry, agriculture and medicine. *Fusarium oxysporum* is a worldwide distribution sections of plant pathogenic fungi, whose host is range, can cause melons, bananas, solanaceae, more than 100 kinds of plants such as cotton, leguminosae and flowers to blight [2]. Fusarium wilt, caused by the *F. oxysporum* sp., which is one of the most destructive diseases that can afflict banana, potato, tomato and crown plants. It is both necessary and urgent to find an efficient method for protecting food production worldwide [3] [4].

Antifungal peptides are one of the most important natural defenses, which were against the invading of most fungal pathogens. Some has been developed to be the food preservatives and bio-pesticides, which has provided a new choice to prevent and control the fungal contamination of agricultural products. Studies have focused on the application of lipopeptides in the prevention and control of pathogens [5] [6] [7]; Several *Bacillus* strains produce biologically active compounds, including lipopeptides, with an evident effect on plant disease control, including *Fusarium* [8] [9]. *Bacillus amyloliquefaciens* NJN-6 produced volatile compounds (VOCs) that inhibited the growth and spore germination of *F. oxysporum f.* sp. *cubense* [10]. Lipopeptides produced by *B. amyloliquefaciens* Q-426 showed antifungal activity against *F. oxysporum f.* sp. *spinaciae* [11] [12]. Researchers also isolated the active substance from *Bacillus subtilis* JA by reversed phase HPLC separation, identified two iturin A homologs through ESI-CID mass spectrometry analysis and their molecular weight respectively were 1042 Da and 1056 Da. Antifungal activity test showed that *B. subtilis* JA could inhibit wheat scab (*Fusarium graminearum*), watermelon fusarium wilt (*F. oxysporum*) and other various plant pathogens.

The Iturin family that comprises iturin A, C, D and E, bacillomycin D, F and L, bacillopeptin and mycosubtilin [13], which is a kind of cyclic lipopeptide containing 7 amino acids, with strong antifungal activity against *Fusarium* strains [14]. The inhibitory effect of iturin family was observed by researchers in abnormal conidial germination and spore germination of fungi when treated with different extract concentrations [9] [15]. Optical and fluorescence microscopy analyses revealed several morphological changes in conidia and substantial distortions in *F. graminearum* hyphae treated with iturin A [16]. Bacillomycin D exhibited antifungal effect on the mycelium growth, sportulation and spore germination of *Aspergillus flavus*, and it could injure the cell wall and cell membrane of hypha and spore then cytoplasms by observation of SEM and TEM [17]. According to literatures, a very large number of antifungal proteins active on the fungal cell wall, on enzymes of the cell wall synthesis machinery, the plasma membrane and on intracellular targets have been characterized [18]. Hajare *et al.* [19] found that the antifungal activity of bacillomycin D like cyclic lipopeptide produced by *B. amyloliquefaciens* ATCC23350^{T} was due to its inhibitory effect on β-1,3-glucan biosynthesis, a major fungal cell-wall component. In addition, some researchers showed that the Cc-GRP-fungi interaction led to morphological changes and membrane permeability, including the formation of

pseudohyphae, which were visualized with the aid of SYTOX green dye. Cc-GRP coupled to FITC and its subsequent treatment with DAPI revealed the presence of the peptide in the cell wall, cell surface and nucleus of *F. oxysporum* [20]. They also investigated the effect of borate on spore germination of the fungal pathogen *in vitro* and anthracnose control in harvested mango fruit, and observed mitochondrial damage in the spores under borate exposure, in order to evaluate the mechanism of its antifungal action. Baysal *et al.* [4] showed lipopeptides could inhibit the mycelium growth and spore germination of *F. oxysporum*. To elucidate the mechanism of action with cytoplasmic membranes, Hao *et al.* [21] examined the membrane permeability of antimicrobial peptides. However, few report investigated the detail inhibitory effect of iturin A produced by *Bacillus* strains on antagonism of *F. oxysporum* which would be shown in this study.

Bacillus amyloliquefaciens BH072, a novel bacterium isolated from honey sample, showed antifungal activities against mold [22]. One of the antifungal substances was identified to be iturin A [23]. The yield and antifungal activity of iturin A produced by strain BH072 were higher than those of other iturins. RSM has been employed to optimize the components of a medium and the fermentation conditions for cyclic lipopeptide production in shake-flask fermentation [24]. The amount of iturin A was tenfold higher than the production yield in a previous optimization study [25]. In this study, we try to investigate the structure and antifungal effect of this lipopeptide produced by strain BH072, including mycelial growth and spore germination of *F. oxysporum*. MIC of iturin A was determined according to the spore germination and mycelium growth inhibition as well. Few report showed the MIC results of iturin A against *F. oxysporum*, and selecting the concentration of iturin A was important for further tests. The target structures that will be discussed in this study range from the outermost part of fungal well, which is defined by the cell wall, to plasma membrane and finally to several intracellular targets. Results of this study indicated that iturin A had antifungal effects on *F. oxysporum*, and gave rise to the deleterious cellular consequences. The isolated *Bacillus* strain BH072 that can produce large quantities of lipopeptide iturin A has significant potential for use as a biocontrol agent for controlling *Fusarium* pathogens in agricultural production systems.

2. Materials and methods

2.1 Microorganisms and Culture Conditions

Strain BH072 was isolated from a honey sample and identified as *Bacillus amyloliquefaciens* [22] in Luria-Bertani medium (peptone, 10 g; yeast extract, 5 g; NaCl, 18 g; and distilled water, 1 L.) was used to grow BH072. *F. oxysorum* CGMCC 3.2830 was purchased from the China General Microbiological Culture Collection Center and was grown in Potato-Dextrose (PD) medium (potato, 200 g; glucose, 20 g; and distilled water, 1 L). 1.5% agar was added for solid medium PDA if needed.

2.2. Purification and Structure Determination of Iturin A

Bacillus amyloliquefaciens BH072 from a single colony was inoculated into a 500 mL shake flask containing 200 mL of LB medium with shaking at 150 rpm for 60 h at 30˚C. After cultivation, the culture was centrifuged at 4200 rpm for 20 min. The cell-free supernatant was adjusted to pH 2.0 by 6 M HCl and stored at 4˚C overnight for precipitation. The precipitate was collected by centrifugation at 4200 rpm for 20 min at 4˚C and freeze dried. The residue was then extracted with 200 mL of methanol under shaking for 24 h at room temperature. The methanol solution of the antifungal substances was obtained after centrifugation at 4200 rpm for 20 min and examined by LC-MS (Thermo Fisher Corporate, USA). Iturin A in the filtrate was identified by MS at m/z 600 to m/z 1300 according to the molecular weight [12] [23]. The methanol extraction liquid was evaporated in an oven at 60˚C. Then the concentration of iturin A solution was calculated [24]. Final concentration 20 mg/mL solution of iturin A was used for its structure and antifungal mechanism detection.

Methanol extract containing iturin A was dissolved in methylene chloride. Iturin A samples were pointed on two silica thin-layer chromatography (TLC) sheets and unfolded in chloroform, acetic acid = 8:2 (V/V) for 10 min [9]. After the solvent was volatilized, one plate was directly colored with ninhydrin reagent (0.5% of ninhydrin in aceton solution) (plate A). Another plate was put in the high temperature resistant glasswares, fumigated at 110˚C oven for 1 h by acid hydrolysis with 1 mL of high concentrated hydrochloric acid, cooled in the fume hood, and then colored with ninhydrin reagent (plate B).

The FT-IR spectrum of iturin A was recorded using KBr pellet in a Bruker Tensor 27 system. Dried sample was prepared by dispersing the solid uniformly in a matrix of dry nujol (KBr) mull, compressed to form an almost transparent disc [26]. IR spectra were collected from 400 - 4000 wave numbers (cm^{-1}).

2.3. Effects of Iturin A on Spore Germination and Hyphae Growth of *F. oxysporum*

F. oxysporum cultures were transferred to Petri dishes containing PDA plate for 6 days; spores were added to MiliQ water, and gently agitated for 1 min for liberation. After plated on PDA, spores were quantified to determine the appropriate dilutions [20]. The 20 mg/mL iturin A solution was serially double diluted with MiliQ water up to 128 times. 1 mL diluted iturin A sample was uniformly added into 9 mL PDA medium at 40˚C - 50˚C and poured into Petri dishes. PDA plates without iturin A were used as control.

100 uL of 1.0×10^6 cells/mL spore suspension was spread on the PDA plates contains series concentration of iturin A. Spores were counted after incubation at 28˚C for 24 h [17] [27]. Each treatment was determined in triplicate and the experiments were repeated twice. The inhibition rate of iturin A on spore germination of *F. oxysporum* was calculated by the following formula: Inhibition rate (%) = (1 − spore's number of iturin A plate/spore's number of control) ×

100%.

The inhibitory effects on hyphae growth was determined by growth rate assay. 6 days old hyphae discs (5 mm) of *F. oxysporum* 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 iturin A was measured by decussation method. Each treatment was determined in triplicate and the experiments were repeated twice. Growth inhibition of indicators was calculated as the percentage of inhibition of diametrical growth relative to the control, of which formula is: Inhibition rate (%) = (1 − the hyphae growth diameter of iturin A plate/plate diameter of control) × 100%.

Minimum inhibitory concentration (MIC) was considered as the lowest concentration of the test compound at which inhibition rates of both spore germination and hyphae growth was higher than 90% [28]. The MIC of iturin A would be determined and used in the later assays that could inhibit the fungal growth.

2.4. Effects of Iturin A on Cell Morphology and Physiology of *F. oxysporum*

The spores of *F. oxysporum* were incubated in liquid PD medium at 150 rpm for 8 h at 28˚C. For microscopic analysis, the fungal culture was then treated with 20 mg/mL iturin A for 48 h. Culture without iturin A treatment was used as control. After centrifuged at 6000 rpm for 5 min, the hyphae grown in PD were harvested. Slides were then prepared from treated and control cultures and observed under a light microscope [19] [29].

Effect of the antifungal lipopetide iturin A on the cell walls was indicated by measuring the activity of alkaline phosphatase (AKP) [30]. In order to obtain the mycelium suspension, the spores were incubated in liquid PD medium shaking at 150 rpm for 48 h at 28˚C. Then iturin A was added to 10 mg/mL (4 * MIC), 5 mg/mL (2 * MIC) and 2.5 mg/mL (1 * MIC) into each fungal culture and 1 mL culture sample was taken out at 0, 2, 4, 6, 8, 12, 24 and 48 h time point, respectively. The culture without adding antifungal substances was performed as control. After centrifuged at 12,000 rpm for 2 min, the supernatant was collected as the enzyme sample to test the activity of AKP. The detection method was described as follows. 0.1 mL 20 mmol/L p-Nitrophenyl Phosphate Disodium (pNPP) was added into 1.8 mL 0.1 mol/L Na_2CO_3-$NaHCO_3$ buffer (pH 9.7), and then the solution was heated for 10 min at 37˚C. Each 0.1 mL enzyme sample was respectively mixed into the above-mentioned solution for 10 min at 37˚C, while 0.1 mL MiliQ adding into the same solution was used as blank control. 1 mL 0.5 mol/L NaOH was used to terminate the enzymatic reaction, and then the absorbance of AKP was measured at 405 nm.

2.5. Effects on Cell Membrane and Cell Proteins of *F. oxysporum*

In order to obtain the mycelium suspension, the spores were incubated in liquid PD medium shaking at 150 rpm for 48 h at 28˚C. Mycelium was collected by

centrifugation at 6000 rpm for 5min. Each 1.5 g mycelium was added into concentration of 10 mg/mL (4 * MIC), 5 mg/mL (2 * MIC) and 2.5 mg/mL (1 * MIC) iturin A solution, then cultivated together at 28˚C, 150 rpm. Culture samples were taken at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h, respectively. And conductivity was measured for each time point sample. Sample with no antifungal substance was used as blank control. Conductivity was measured by conductivity meter (DDS-307, Shanghai Precision Scientific Instrument Co., Ltd. Shanghai, China).

Malondialdehyde (MDA) is one of the products of cell membrane lipid peroxidation, and membrane lipid oxidation degree index can aggravate the damage of membrane [31] [32]. Mycelium were collected by centrifugation at 6000 rpm for 5 min after 48 h spores' cultivation the same as above. Each 0.5 g mycelium was added into concentration of 10 mg/mL (4 * MIC), 5 mg/mL (2 * MIC) and 2.5 mg/mL (1 * MIC) iturin A solution, then cultivated together at 28˚C, 150 rpm. Each 0.5 g mycelium as samples was taken on ice after 48 h incubation. In order to obtain completed cell membrane ingredient, 2 ml 0.05 mol/L PBS buffer was added to each sample for suspension. Then they were heated with 5 mL 0.5% glucosinolates barbituric acid (TBA) for 10 min, and immediately moved on ice. After centrifuged at 5000 rpm for 10 min, the supernatant was collected for testing OD at 450 nm, 532 nm and 600 nm and its volume was measured. Then MDA concentration would be calculated by the following formula: MDA (mmol/g * Fw) = [6.452 * (A532 − A600) − 0.559 * A450] * Vt/(Vs * Fw) [33].

Some spores of *F. oxysporum* were inoculated into PD medium shaking at 28˚C, 150 rpm. When the mold spore's liquid turned to be turbid (12 h cultivation), iturin A was added to this solution to final concentration of 10 mg/mL (4 * MIC), 5 mg/mL (2 * MIC) and 2.5 mg/mL (1 * MIC) and started the time, then each 1 mL fungal culture was taken after 48 h cultivation. Then the supernatant was harvested by centrifugation at 12,000 rpm for 10 min and dried by freeze-drying. The powder dissolved in TE buffer was used as the extracellular protein solution. Then the protein concentration of extracellular samples was tested by coomassie brilliant blue method. The harvest cell pellets were washed twice by PB buffer (50 mmol/L Tris-HCl pH 8.0, 100 mmol/L NaCl) and then resuspended in 5 mL of PB buffer, respectively. The mixture was ultrasonic disrupted 10 s with 20 s pauses at 200 HZ for 30 times. The cell solution was centrifuged at 6000 rpm for 10 min and the supernatant was collected as intracellular protein solution. The intracellular proteins solution was detected by sulfate-polyacryamide gel elecrophoresis (SDS-PAGE) [34].

3. Results

3.1. Structure Analyses of Iturin A

3.1.1. Cyclic Structure Analyses of Iturin A by TLC

Cyclic lipopeptide molecule amino acids are all involved in forming rings. No N-terminal exposes and no ninhydrin coloration occurs. However, if cyclic lipopeptide samples reacted with situ acid, ninhydrin coloration will occur with

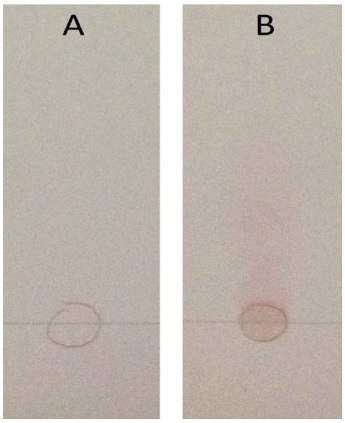

Figure 1. TLC of Ninhydrin coloration. Plate A: colored with ninhydrin reagent (0.5% of ninhydrin in aceton solution). Plate B: put in the high temperature resistant glasswares, 110˚C oven fumigation for 1 h by acid hydrolysis, then colored with ninhydrin reagent.

yellow or purple spots appearing. Results shown in **Figure 1** indicated that there were a few purple spots on B plate (in a few cases some display yellow spots), while no significant splash on A plate point in the corresponding position, suggesting that the peptide sample may contain a cyclic peptide or peptide amide. Accordingly it could be determined that the sample is a cyclic lipopeptide compound.

3.1.2. Fourier Transformed-Infra Red (FT-IR) Spectra of Iturin A

FT-IR spectrum and its derivative spectrum of iturin A were showed below in **Figure 2**. All the valid corresponding fractions could be figured out from both spectrums. For all fractions, the FT-IR spectrum of iturin A analysis showed bands at 3400 cm^{-1} (-NH), 1543 cm^{-1} (the deformation mode of the N-H bond combined with C-N stretching mode (amide II band)) and 1655 cm^{-1} (the stretching mode of the CO-N bond (amide I band)), indicating the presence of a peptide component. There were also bands at 2855 - 2959 cm^{-1} and 1339 - 1449 cm^{-1}, resulting from typical C-H stretching vibration in the alkyl chain. The band at 1236 cm^{-1} and 1720 cm^{-1} was due to lactone carbonyl absorption, which indicated that the sample was a kind of lipopeptide substance. According to reports, the secondary structure of amide I peaks was identified by the following: 1615 - 1638 cm^{-1} is β-fold, 1638 - 1645 cm^{-1} is random coil, 1645 - 1662 cm^{-1} is α-helix, 1662 - 1695 cm^{-1} is β-corner. The secondary structure of the amide II peaks identified not yet seen reports. In this study, iturin A should have structure of α-helix with a peak at 1655 cm^{-1}.

3.2. Effect of Iturin A on *F. oxysporum* by Morphological Observation

3.2.1. Microscopic Examination

Microscopic examination of the fungus revealed that treatment with the iturin A caused abnormal mycelial growth, as evidenced by increases in mycelial apex offshoots, distortion and tumescence. As shown in **Figure 3**, control hyphal cells were intact, smooth and had a fine structure. After treating with iturin A for 48 h,

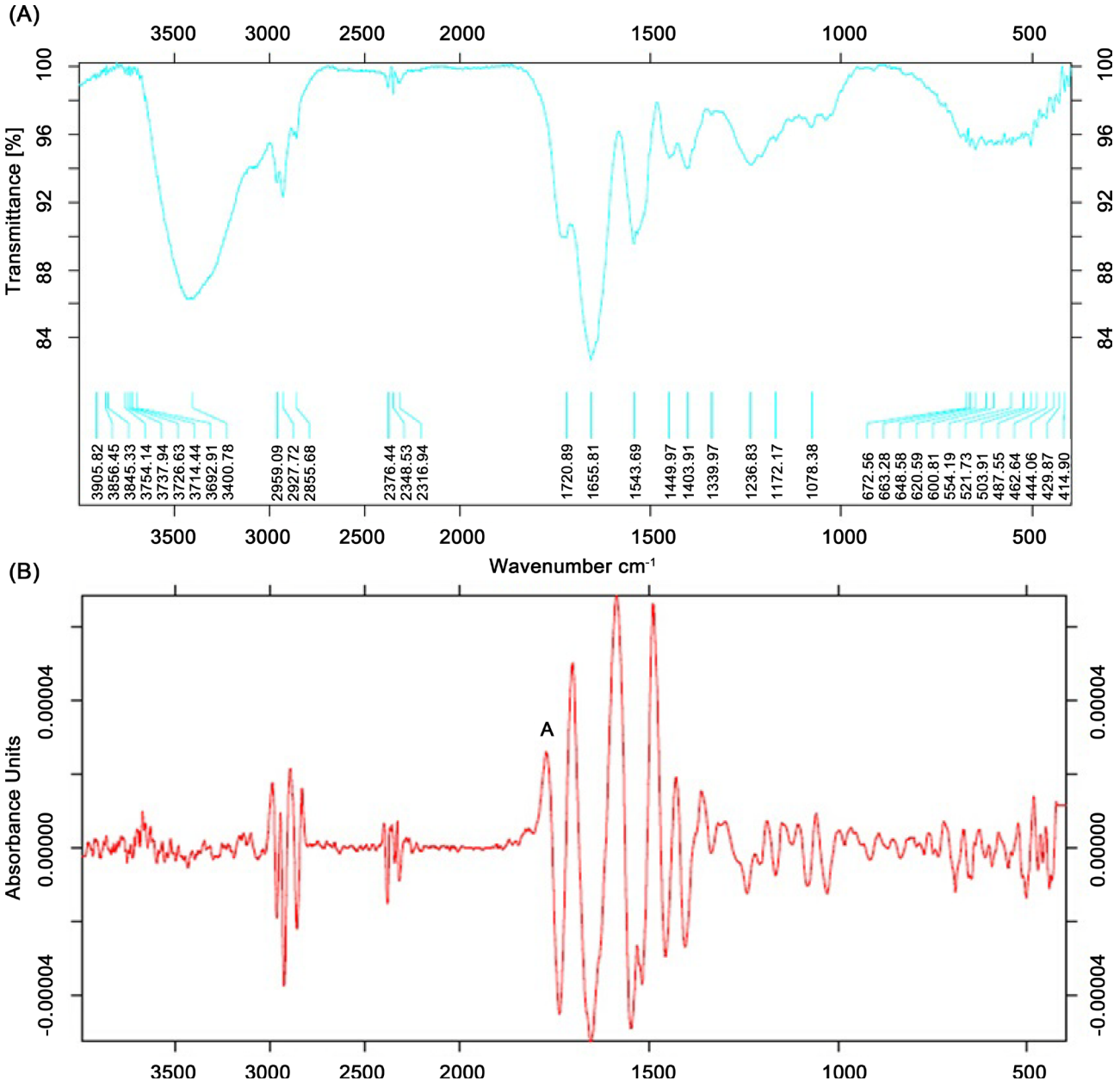

Figure 2. (A) Fourier transformed infrared spectra of iturin A; (B) Derivative spectrum of FT-IR. Sample preparation in KBr tablets was recorded from 400 - 4000 cm^{-1}.

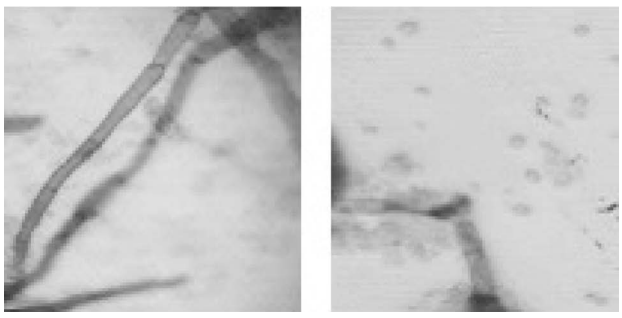

Figure 3. Morphology of control and iturin A-treated *F. oxysporum* hyphae under light microscope. A. Control mycelium, not treated with iturin A; B. Test mycelium after being treated with the iturin A (20 mg/mL).

the surface of the hyphal cells was rough with a lumpy appearance and abnormal configuration. These features appeared frequently in cells.

3.2.2. Effect of Iturin A on Spore Germination and Hyphae Growth of *F. oxysporum*

The spore numbers of *F. oxysporum* formed on plates gradually reduced with increasing the inhibition rate related to the concentration of iturin A when cultivated in PD medium. Results investigated that iturin A significantly inhibited the formation of the spores of *F. oxysporum*. 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 spores when they were incubated in the PD medium. After incubation of 24 h, most spores without iturin A treatment (the control) germinated, the spore balls expanded and the germ tube had been formed. When the iturin A concentration was diluted by 8 times, about 2.5 mg/mL, the inhibition rate was found to be 96.36%. While for higher dilutions, inhibition rates were decreased gradually to 87.79% at 16 times dilution, then down to 19.48% at 128 times dilution. It was obvious that the iturin A concentration for completed inhibiting spore forming and spore germination was 2.5 mg/mL or more, when inhibition rates was higher than 90%.

According to the data from Table 1, iturin A could obviously inhibit the hyphae of *F. oxysporum*. When the iturin A concentration was 2.5 mg/mL, the inhibition rate to hyphae reached to 90.33%. The higher the iturin A concentration, the more the inhibition rate to hyphae growth was. When the iturin A concentration was set at 5.0 mg/mL, the inhibition of hypha growth was found to be 97.44%. As the hyphae of fungi was already formed, more iturin A was needed to inhibit their growth than adding from the spore formulation stage. For completed inhibiting hyphae growth and spore germination at the same time, the concentration of iturin A was 2.5 mg/mL or more. And the MIC of iturin A against *F. oxysporum* should be 2.5 mg/mL, when both 90% spore germination and hyphae growth could be inhibited.

3.3. Effect of Iturin A on Cell Wall of *F. oxysporum*

Normally AKP exists between the cell membrane and cell wall, if the cell wall was damaged, the activity of AKP in culture would increase. Results indicated

Table 1. The effect of iturin A on the inhibition of spore germination and hyphae growth.

Concentration of iturin A (mg/mL)	20	10	5	2.5	1.25	0.625	0.3125	0.15625	0 (Control)
Spores number	0	0	5	14	47	61	118	310	385
Inhibition rates of Spore germination (%)	100	100	98.70	96.36	87.79	84.16	69.35	19.48	0
Hyphae growth diameter (mm)	90	90	2.3	8.7	44.2	67.0	78.4	80.1	90
Inhibition rates of Mycelial growth (%)	100	100	97.44	90.33	50.89	25.44	12.89	11.00	0

that by iturin A treatment, the cell wall of *F. oxysporum* was destructed by markedly observed improvement of the activity of AKP (Figure 4). The AKP activity of fungal culture increased with the increasing concentration of iturin A. After the treatment of iturin A for 12 h, the activity of AKP did not increase any more. It also indicated that the destruction of cell wall could be one of the important mechanisms of the antifungal effect.

3.4. Effect of Iturin A on Cell Membrane of *F. oxysporum*

3.4.1. Conductivity Measurement

According to the literatures, if the membrane system was destroyed, the cell membrane permeability would increase. The scale of the membrane permeability could be detected by measuring electric conductivity of fungal culture, in order explore whether the membrane system was damaged or not. The electric conductivity increased over time until the constant was not variable. Obviously, the higher concentration of iturin A, the bigger electric conductivity could be tested (Figure 5). So iturin A treatment could cause the leakage of cytoplasm, making electric conductivity increase, leading to *F. oxysporum* cell membrane destroyed.

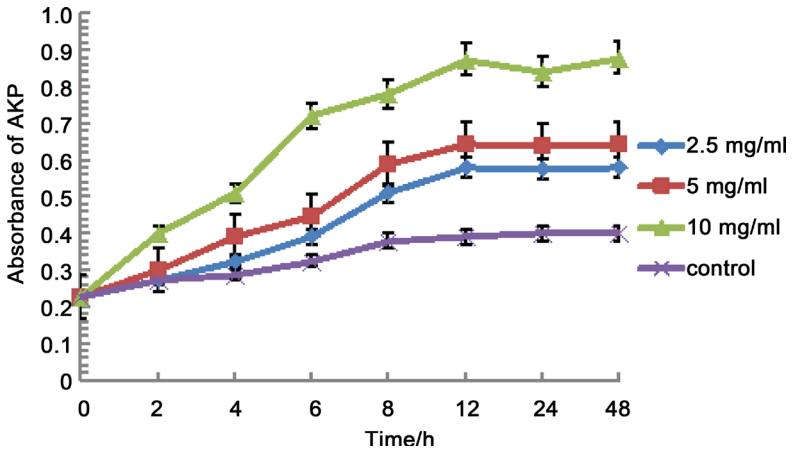

Figure 4. Activity of AKP of *F. oxysporum* culture treated with different concentration of iturin A.

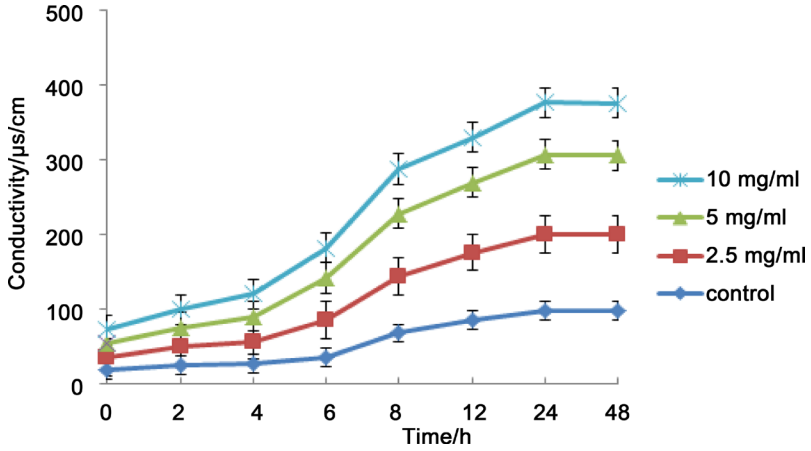

Figure 5. Conductivity of *F. oxysporum* culture treated with different concentration of iturin A.

3.4.2. MDA Measurement

As a result, concentration of MDA of *F. oxysporum* culture increased after treated with iturin A for 48 h. The higher the iturin A concentration, the more MDA was tested, which represented the level of membrane lipid peroxidation occurred, and also indirectly reflected *F. oxysporum* cell membrane was damaged. When MDA was heated under acid condition with glucosinolates barbituric acid (TBA), colorful product would be produced, which was pink 3,5,5-three oxazole, 2,4-dione (Trimet-nine). This material could be detected under the 532 nm. TBA can react with other substances, resulting in the wavelengths are absorbed. In order to eliminate sulfur barbituric acid reaction with other substances, the absorbance of 600 nm was measured at the same time, then MDA were calculated by using the 532 nm and 600 nm absorbance (Table 2).

3.5. Effect of Iturin A on Cell Proteins of *F. oxysporum*

The extracellular protein concentration increased by adding iturin A (shown in Figure 6), which might result from the damaged membrane. Compared with control, intracelluar protein electrophoresis bands faded with treatment of increasing concentration of iturinA, indicating that iturin A had effect on normal metabolic of *F. oxysporum*. Combined with extracellular and intracellular protein

Table 2. MDA concentration calculation.

Vt = 10 mL	Iturin A			
	Control	2.5 mg/mL	5 mg/mL	10 mg/mL
Vs/mL	5.0	5.0	5.0	5.0
Fw/g	0.5	0.5	0.5	0.5
A532	0.175	0.171	0.144	0.141
A600	0.131	0.086	0.048	0.047
A450	0.413	0.353	0.3	0.248
MDA (mmol/g)	0.2121	1.4044	1.8068	1.8714

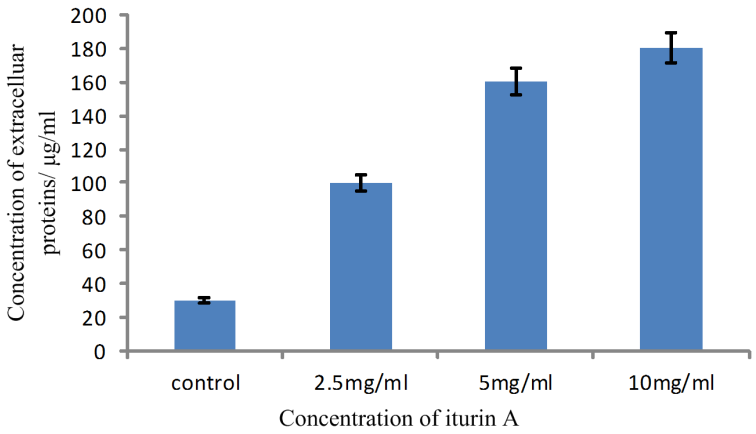

Figure 6. Concentration of *F. oxysporum* extracelluar proteins treated with different concentration of iturin A.

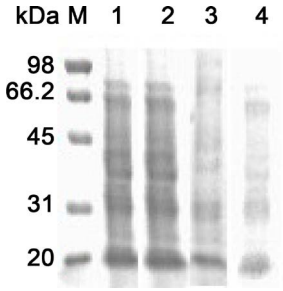

Figure 7. SDS-PAGE of *F. oxysporum* intracelluar proteins treated with different concentration of iturin A. Lane 1. Treated without iturin A; Lane 2. Treated with 2.5 mg/mL iturin A; Lane 3. Treated with 5 mg/mL iturin A; Lane 4. Treated with 10 mg/mL iturin A.

detection, iturin A treatment resulted in the fungal overall total protein levels decreased (**Figure 7**).

4. Discussion

Bacillus strains are the important biocontrol agents and lipopeptides produced by them suppresses several fungal pathogens of plants [35]. Iturin A is one of the effective inhibitors in the biocontrol activity of *B. amyloliquefaciens* BH072 isolated from honey in previous work. As a supplement for iturin A identification, we elucidated its cyclic structure and secondary structure. An α-helical structure related to its antifungal activity was predicted in iturin A compound by FI-TR which has never been reported before. There is an amphiphilic character of iturin A, thus pointing towards the cellular membranes as the most probable site of its action [36]. It showed an effective inhibitory effect on *F. oxysporum*, suggesting that antibiosis may be involved in the disease control and potential for agricultural application.

Some reports investigated that iturin A had biocontrol activity against several *Fusarium* pathogens, such as *F. oxysporum*, *F. aromaticum*, *F. graminearum* and *F. moniliforme* [37]. Here we firstly studied the antifungal effect of purified iturin A on *F. oxysporum* fungal cell wall, cell membrane and cell contents. From morphological observation, iturin A could injure cells of hyphae and spores and damage cell membrane, which are same as surfactin and fengycin [17]. In this study, the AKP activity, conductivity and protein contents were measured; and results suggested that iturin A undermined the integrity of the fungal cell wall and membrane, causing leakage of the contents. Antibiotics in the iturin family were found to act upon sterols presenting in the cytoplasmic membrane of microorganisms [38]. Although cell membrane was the most significant defense and prime sites for antimicrobial attack, destroy of fungal cell wall was also seriously targeted for antifungal activity. From both microscope observation of hyphae and activity of AKP, the cell wall of *F. oxysporum* was destroyed. Due to the damage of cell wall, cell membrane was easily attacked by iturin A. The electric conductivity in the PD culture media all significantly increased with the incubation time after iturin A treatment, which determined that the membrane permeability increased. And extracelluar and intracellular proteins also influenced

by treament of iturin A. Since MDA was always used to test the degree of plants membrane lipid peroxidation, MDA measurement was conducted to explore whether membrane of fungi was peroxided or not under the treatment of iturin A. Sensitive fungi demonstrated a loss of fatty acid unsaturation, which was accompanied by an elevation in MDA [39]. Peroxidation of fungal cell membrane causes increasing membrane fluidity by disturbing hydrophobic phospholipids [40] [41]. The potential mechanism of antifungal activity of iturin A against *F. oxysporum* should be combination of cell wall destroy, cell membrane permeability increasing, and cell membrane peroxided, resulting in cell contents flowing out.

In order to study the MIC of iturin A extracted from *B. amyloliquefaciens* BH072 against *F. oxysporum*, different concentration of iturin A was used to test the effects of spore germination and hyphae growth of *F. oxysporum*. MIC of 2.5 mg/mL iturin A extract for *F. oxysporum* was determined which could inhibit more than 90% spore germination and hyphae growth simultaneously. According to literatures, MICs of iturin A for other pathogens were estimated as well. Iturin A produced by *B. amyloliquefaciens* PPCB004 showed strong inhibition displaying a MIC of 1.0 mg/mL for *F. aromaticum*, 1.5 mg/mL for *Botryosphaeria* sp., and 3.5 mg/mL for *P. crutosum* and 6.0 mg/mL for *P. persea* [15]. MIC tests showed that iturin A produced by *B. amyloliquefaciens* S76-3 at 0.05 mg/mL completely inhibit *F. graminearum* conidia germination [15]. We considered the inhibition rate of hyphae growth following the observation of hyphae damaged. More iturin A was needed to inhibit hyphae growth at the same concentration because spores were already germinated. Compared with other lipopeptides, iturin A was active at higher concentration may result from its structural properties: shorter fatty acid tail. In the previous work, production of iturin A was optimized by response surface methodology. The amount of iturin A produced by strain BH072 was tenfold higher than the production yield in a previous optimization study [24]. Combined with the yield and MIC of iturin A, the results reported here indicated that iturin A plays a vital role in the antifungal activity of *B. amyloliquefaciens* BH072 against *F. oxysporum*.

5. Conclusion

As a conclusion, the antifungal compound iturin A, which had already been identified, was characterized as a cyclic lipopeptide in this study, and displayed antifungal activity against *F. oxysporum* with a MIC of 2.5 mg/mL. The antifungal activity of iturin A was facilitated by the co-function including damage of fungal cell wall, cell membrane and cell contents. Taken together with previous work, iturin A and its producer strain *B. amyloliquefaciens* BH072 might be powerful tools in food and plant pathogens protection.

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