Isolation, Identification, and Antibacterial Activity of Fengycin, an Antimicrobial Lipopeptide from Bacillus amyloliquefaciens TF28 (Postprint)

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Abstract

This study investigates the isolation, identification, and antifungal activity of antimicrobial lipopeptides produced by Bacillus amyloliquefaciens TF28. Crude antimicrobial lipopeptide extracts were prepared using acid precipitation, ethyl acetate, and methanol extraction techniques. Following two rounds of HPLC separation and purification, eight purified antimicrobial lipopeptide fractions were obtained within a retention time range of 32-42 min. These compounds were identified as fengycins by MALDI-TOF-MS and exhibited strong antifungal activity against Fusarium oxysporum and Fusarium graminearum. This research establishes a foundation for directed genetic modification to enhance antimicrobial lipopeptide production in strain TF28.

Full Text

Isolation and Identification of Antifungal Lipopeptide Fengycin Produced by *Bacillus amyloliquefaciens* TF28 and Its Antifungal Activity

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Abstract: This study focused on the isolation, identification, and antifungal activity of antimicrobial lipopeptides produced by a *Bacillus amyloliquefaciens*

TF28 strain. Antimicrobial lipopeptide crude extracts were prepared using acid precipitation, ethyl acetate, and methanol extraction techniques. After two rounds of high-performance liquid chromatography (HPLC) separation, eight antimicrobial lipopeptides were obtained within a retention time of 32–42 minutes and identified as fengycins by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). These compounds demonstrated strong antifungal activity against *Fusarium oxysporum* and *Fusarium graminearum*. These findings establish a foundation for the directed genetic modification of strain TF28 to improve antimicrobial lipopeptide yield.

Keywords: *Bacillus amyloliquefaciens*; *Fusarium oxysporum*; *Fusarium graminearum*; antifungal lipopeptides; fengycin

Introduction

Bacillus amyloliquefaciens is widely distributed in nature and represents an important biocontrol resource that has attracted considerable attention from researchers worldwide due to its ability to secrete abundant antimicrobial substances. Current research on the isolation, purification, and application of antimicrobial substances from *B. amyloliquefaciens* has become a hotspot in the field of biological control [1-5]. This species can produce various antimicrobial active substances, including peptides, lipopeptides, and proteins, with types and yields varying by strain. Studies have shown that the primary lipopeptide antimicrobial substances produced by *B. amyloliquefaciens* include iturin, fengycin, and surfactin, which are synthesized via non-ribosomal pathways by multi-enzyme complexes. Each lipopeptide typically has 3–5 homologues that differ in fatty acid chain length [6]. Due to their diverse structures, broad-spectrum antimicrobial activity, and excellent stability, these compounds participate in multiple biocontrol processes, such as antagonizing pathogens, promoting rhizosphere colonization of biocontrol agents, and inducing systemic resistance in plants, thereby playing important roles in plant disease biological control [7-10].

Strain TF28 is an endophytic bacterium isolated from soybean roots with broad-spectrum antimicrobial activity [11]. Genome sequencing revealed that this strain contains three types of lipopeptide biosynthetic gene clusters [12]. Previous studies demonstrated that TF28 secretes iturin lipopeptides [13]. To determine whether the strain produces other lipopeptide types and to lay a foundation for strain application and genetic modification, this study investigated the isolation, purification, and antifungal function of fengycin.

Materials and Methods

1.1 Test Strains

Bacillus amyloliquefaciens TF28 was isolated from soybean roots by the Institute of Microbiology, Heilongjiang Academy of Sciences, and maintained on

LB medium. *Fusarium oxysporum* (strain HWS04) and *Fusarium graminearum* (strain HWS07) were preserved in the Heilongjiang Provincial Key Laboratory of Bioengineering and maintained on potato dextrose agar (PDA) medium.

1.2 Extraction of Antimicrobial Lipopeptides

Antimicrobial lipopeptide crude extracts were obtained using acid precipitation. A loopful of activated TF28 cells was inoculated into 3 mL of LB liquid medium and cultured at 30°C with shaking at 200 r/min for 16 h. The culture was then transferred to 200 mL of optimized lipopeptide production medium [14] (containing glucose 42.37 g/L, yeast extract 2 g/L, beef extract 2 g/L, ammonium sulfate 2 g/L, magnesium sulfate 2.11 g/L, calcium chloride 0.1 g/L, manganese sulfate 0.1 g/L, potassium dihydrogen phosphate 1.5 g/L, and disodium hydrogen phosphate 3 g/L) at a 2% inoculation ratio, and incubated at 30°C with shaking at 200 r/min for 48 h. The culture was centrifuged at 8000 r/min for 20 min at 4°C, and the supernatant was collected. The pH was adjusted to 2.0–2.5 with 5 mol/L HCl and stored overnight at 4°C. After centrifugation at 8000 r/min for 20 min, the precipitate was collected, air-dried, extracted once with methanol, and then subjected to ethyl acetate/water partitioning. The organic phase was evaporated to dryness, and the residue was dissolved in methanol to obtain the antimicrobial lipopeptide crude extract.

1.3 Separation and Purification of Fengycin Lipopeptides

The crude lipopeptide extract was filtered through a 0.22 m membrane and separated using a Shimadzu HPLC system equipped with a C18 reversed-phase column. Mobile phase A consisted of water/TFA, and mobile phase B consisted of methanol/TFA. The elution gradient increased mobile phase B from 30% to 70% over 10 minutes, then to 100% over 25 minutes. HPLC fractions were manually collected, concentrated using a rotary evaporator, and tested for antifungal activity. Active fractions were subjected to a second round of HPLC purification, and the resulting fractions were again tested for activity.

1.4 Determination of Antifungal Activity

Antifungal activity of crude extracts and HPLC-purified fractions was evaluated using the paper disc diffusion method. A fungal plug (8 mm diameter) was placed in the center of a 90 mm PDA plate, and sterile filter paper discs were positioned 2 cm from the fungal plug. Ten microliters of sample were applied to each disc, with solvent serving as the control. Plates were incubated at 28°C for 3–5 days and examined for antifungal activity.

1.5 Mass Spectrometry Analysis

Active fractions were analyzed by MALDI-TOF-MS. The CHCA (-cyano-4-hydroxycinnamic acid) matrix was dissolved in 30% acetonitrile containing 0.1% TFA. Spectra were acquired in positive ion reflectron mode. Based on literature

reports of *Bacillus* lipopeptide antibiotics and their molecular weights, the antifungal substances in TF28 were identified.

Results

2.1 Antifungal Activity of Crude Lipopeptide Extract

Using *F. oxysporum* and *F. graminearum* as indicator fungi, the antifungal activity of the crude lipopeptide extract was evaluated. As shown in [Figure 1: see original paper], the lipopeptide extract inhibited the growth of both pathogenic fungi, with stronger activity against *F. oxysporum*.

2.2 Separation and Purification of Antimicrobial Lipopeptides

The crude lipopeptide extract was subjected to initial HPLC separation, yielding multiple peaks within 60 minutes. Eleven fractions were collected ([Figure 2: see original paper]). Antifungal activity testing against *F. oxysporum* revealed that three fractions (1-5, 1-6, and 1-7) exhibited significant inhibition ([Figure 3: see original paper]), with retention times between 32–42 minutes. These three fractions were individually subjected to a second purification step. Fractions 1-5 and 1-6 each yielded a single major peak (2-1 and 2-2, respectively), while fraction 1-7 produced multiple peaks (2-3, 2-4, 2-5, 2-6, 2-7, and 2-8) ([Figure 4: see original paper]). All collected fractions were tested for antifungal activity. The results showed that fractions 2-1, 2-2, 2-3, 2-4, 2-5, and 2-6 strongly inhibited both pathogens. Fraction 2-8 showed strong activity against *F. graminearum* but weaker activity against *F. oxysporum*, while fraction 2-7 exhibited relatively low activity against both fungi ([Figure 5: see original paper]).

2.3 MALDI-TOF-MS Analysis of Antimicrobial Lipopeptides

MALDI-TOF-MS analysis was performed on the eight purified active fractions from the second purification step. Primary mass spectrometry revealed that fractions 2-1, 2-2, 2-3, 2-5, and 2-7 ([M+H] at m/z 1463.7832, 1491.8247, 1505.8154, 1491.7981, and 1519.8358) belonged to the first homologue group, while fractions 2-4, 2-6, and 2-8 ([M+H] at m/z 1447.7803, 1475.8151, and 1489.8183) belonged to the second homologue group ([Figure 6: see original paper] A-H,).

The molecular masses of the first homologue group differed by 14 Da, corresponding to exactly one CH unit, suggesting either a difference of one methylene group in the fatty acid chain or substitution of Ala by Val at position 6 of the peptide chain. These masses are consistent with previously reported fengycin lipopeptides [15]. Fengycin molecular structure typically comprises a -hydroxy fatty acid and a cyclic decapeptide [16], and is classified as fengycin A or B based on the amino acid at position 6: Ala indicates fengycin A, while Val indicates fengycin B [17]. Identification of fengycin compounds primarily relies on characteristic molecular and fragment ions in mass spectra [17].

Secondary mass spectrometry analysis of the five molecular masses in the first homologue group yielded characteristic fragment ions. Fractions 2-1 and 2-5 showed characteristic ions at m/z 966 and 1080 ([Figure 7: see original paper] I and M,), consistent with fengycin A [17]. Fractions 2-2, 2-3, and 2-7 showed ions at m/z 994 and 1108 ([Figure 7: see original paper] J, K, and O,), matching fengycin B [17].

The second homologue group ([M+H] at m/z 1447.7803, 1475.8151, and 1489.8183) also showed 14 Da mass differences. Secondary mass spectrometry revealed that fraction 2-4 produced characteristic ions at m/z 966 and 1080 ([Figure 7: see original paper] L,), consistent with fengycin A [17], while fractions 2-6 and 2-8 yielded ions at m/z 994 and 1108 ([Figure 7: see original paper] N and P,), characteristic of fengycin B [17]. Therefore, the eight active fractions obtained after the second purification belong to a single class of lipopeptide antibiotics: fengycin.

Discussion

Lipopeptide antimicrobial substances exhibit broad-spectrum resistance against bacteria, fungi, tumors, and viruses. Their stable structure and low potential for resistance development make them ideal antibiotic alternatives with significant application potential in agriculture and medicine. *Bacillus* species are important microbial resources for producing antimicrobial lipopeptides, though most reports focus on isolation and activity characterization. Low production yields have limited their agricultural applications. The types and activities of antimicrobial lipopeptides vary by strain. Li et al. [18] isolated surfactin and fengycin from *Bacillus subtilis* BAB-1, with fengycin inhibiting *Botrytis cinerea* growth. Ji et al. [19] purified fengycin from *B. subtilis* E1R-j that inhibited *Gaeumannomyces graminis* var. *tritici*. In this study, we isolated eight fengycin A and B homologues from strain TF28 that inhibited *F. oxysporum* and *F. graminearum*. The antifungal spectrum of the fengycin obtained in this study differs from literature reports, suggesting structural variations among fengycin homologues produced by different strains.

Fengycin acts on the lipid layer of pathogen cell walls, disrupting membrane structure and permeability, thereby exerting strong inhibitory effects against various phytopathogenic fungi, particularly filamentous fungi. Studies have shown synergistic effects among different lipopeptides. Iturins and fengycins strongly inhibit phytopathogenic fungi [20, 21], while surfactin alone shows limited antifungal activity but enhances the activity of iturins or fengycins [22, 23]. Additionally, surfactin and fengycin can induce systemic resistance in plants (ISR) [24, 25].

The genome of strain TF28 contains gene clusters encoding three types of antimicrobial lipopeptides [12]. Iturin A was previously isolated and shown to inhibit *Fusarium fujikuroi* [13]. Using a non-isocratic HPLC elution gradient, this study successfully isolated eight fengycin homologues, demonstrating that

TF28 produces diverse fengycin variants. However, the yield remains low for practical applications. Based on these results, future work will focus on genetic modification of strain TF28 to enhance lipopeptide production, providing valuable strain resources for antimicrobial lipopeptide applications.

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Note: Figure translations are in progress. See original paper for figures.