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NOTE

Antifungal Activity and Mechanism of Fengycin in the Presence and Absence of Commercial Surfactin Against *Rhizopus stolonifer*

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The antifungal activity and mechanism of fengycin in the presence and absence of commercial surfactin against *Rhizopus stolonifer* were investigated. The MIC (minimal inhibitory concentration) of fengycin without commercial surfactin added was 0.4 mg/ml while the MIC of fengycin with commercial surfactin added was 2.0 mg/ml. Fengycin acted on cell membrane and cellular organs and inhibited DNA synthesis. The antifungal effect of fengycin was reduced after commercial surfactin was added. All these results suggest that the fungal cell membrane may be the primary target of fengycin action and commercial surfactin may reduce the antifungal activity of fengycin.

***Keywords*: *Rhizopus stolonifer*, lipopeptide, antifungal activity, mechanism, fengycin, commercial surfactin**

Rhizopus stolonifer is the causal agent of soft rot in many fruit and vegetables (Zhang *et al*., 2007). *Rhizopus* rot caused by *R. stolonifer* can greatly limit the storage period and market life of fruit and vegetables (Karabulut and Baykal, 2002). This fungus infects ripe fruit after harvest, unless fruit in the field have already sustained major injuries or split pits (Förster *et al*., 2007) and can cause severe agricultural damage in both pre- and post-harvest. For several years, synthetic fungicides have been used to control this microorganism. However, it has been shown that the compounds used in these fungicides can cause strain resistance which would lead to potential risk for the environment and human health (Northover and Zhou, 2002). Therefore, attempts have been made to develop safe, novel, and effective antifungal compounds against this fungus.

Bacillus species have the interesting property of producing secondary metabolites with a wide spectrum of antibiotic activity and very diverse structures. *Bacillus subtilis* strains produce gene-encode antibiotics as well as a variety of small antibiotic peptides synthesized non-ribosomally. These include lipopeptide antibiotics such as fengycin (Vanittanakom *et al*., 1986) and surfactin (Kluge *et al*., 1988). Members of the fengycin and surfactin groups are composed of one hydroxy fatty acid and ten (fengycins) or seven (surfactins) amino acids. The primary function of fengycin is to inhibit the growth of filamentous fungi (Vanittanakom *et al*., 1986). Surfactin is capable of inhibiting the growth of bacteria, viruses and cruor (Singh and Cameotra, 2004), but has no inhibition effects on filamentous fungi.

In order to develop novel bioactive peptides, the mechanisms of action are essential to be understood. Because of the amphiphilic nature of fengycin and surfactin, with a polar amino acid head and a hydrocarbon chain, their main target is the cell membrane where they act to increase cell permeability inducing the cell dye (Maget-Dana *et al*., 1992; Deleu *et al*., 2008). In our previous work, we found that *Bacillus subtilis* fmbJ was capable of producing antimicrobial lipopeptides, fengycin and surfactin (Huang *et al*., 2007). In the meantime, this strain is mainly a high producer of fengycin (Bie *et al*., 2009). There are many reports focused on antifungal activity, structure and synthesis mechanism of fengycin, but few studies have elucidated the antifungal mechanism of fengycin systematically. Furthermore, it has been reported that surfactin may enhance the effect of other lipopeptides (Maget-Dana *et al*., 1992) and the antifungal mechanism of these lipopeptides against *R. stolonifer* has not been elucidated. Therefore, in the present study, the combinative effects of fengycin and commercial surfactin on hypha growth and spore generation of *R. stolonifer* were investigated. The mechanisms in *R. stolonifer* were also studied.

In this study, *R. stolonifer* (AS 3.2336) was obtained from the Institute of Microbiology Chinese Academy of Sciences (IMCAS). Fengycin was produced by fermentation of *B. subtilis* fmbJ as described by Bie *et al*. (2009) except that the fermentation time was 50 h. The purity of fengycin was over 85% and the concentration of surfactin was quite low, which may be neglectable. Commercial surfactin was purchased from Wako Pure Chemical Industries (Japan) and added to the preparation of fengycin. The concentration ratio of fengycin and commercial surfactin was set at 1:1 after commercial surfactin was added.

Determination of the minimal inhibitory concentration (MIC)

The inhibited activity of fengycin with and without commercial surfactin added was determined by microdilution technique

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Table 1. The effect of fengycin with and without commercial surfactin added on the inhibition of hypha growth in *R. stolonifer*

Fengycin concentration (μg/ml)	Fengycin without commercial surfactin added		Fengycin with commercial surfactin added	
	Colony diameter (mm)	Inhibition[a](%)	Colony diameter (mm)	Inhibition[a](%)
Control[b]	73.86±0.52A	0A	74.63±0.46A	0A
25	40.11±0.63B	45.7B	43.66±0.55B	41.5B
50	35.60±0.74C	51.8C	39.55±0.60C	47.0C
100	31.83±0.79D	56.9D	35.45±0.49D	52.5D
200	27.10±0.65E	63.3E	31.12±0.33E	58.3E
400	15.66±0.68F	78.8F	26.05±0.66F	65.1F
600	10.04±0.55G	86.4G	18.21±0.41G	75.6G
800	-	-	14.55±0.57H	80.5H

[a] Inhibition values were calculated using the formula: Inhibition(%)=[1-diametrical growth of treatment (mm) / diametrical growth of control (mm)]×100.
[b] Control indicates no lipopeptide treatment.
Different letters within columns indicate significant differences at P<0.05, according to Tukey's multiple range test.

(Huang *et al*., 2007) on *R. stolonifer*. Results demonstrated that fungal growth couldn't be detected when fengycin without commercial surfactin added was diluted to 0.4 mg/ml, but it could when the concentration was 0.2 mg/ml. Therefore, the MIC of fengycin without commercial surfactin added to spores of *R. stolonifer* was 0.4 mg/ml. In the meantime, the MIC of fengycin with commercial surfactin added was determined to 2.0 mg/ml. Compared the MIC in the presence and absence of commercial surfactin, we could find that the inhibited activity of fengycin decreased when commercial surfactin was added.

Effects of fengycin with and without commercial surfactin added on hypha growth in *R. stolonifer*

The inhibition of hypha growth was determined by growth rate assay. The diameter of the hyphae on each PDA (potato dextrose agar) plate containing lipopeptide was measured when the hyphae in the control plate (no lipopeptide treatment) reached the edges of the plate. Both lipopeptide preparations successfully inhibited the hypha growth of *R. stolonifer* (Table 1). When the fengycin concentration in the two lipopeptide preparations with and without commercial surfactin added was set at 600 μg/ml, the inhibition of hypha growth was found to be 75.6% and 86.4%, respectively. According to previous reports, when the chitosan concentration was set at 3.0 mg/ml, the inhibition of hypha growth in *R. stolonifer* was about 70% (Guerra-Sánchez *et al*., 2008). It is noteworthy that fengycin used in this study has significantly greater antifungal activities against *R. stolonifer* relative to other compounds.

From the results, it was also found that the inhibition of hypha growth by fengycin decreased after commercial surfactin was added, indicating that commercial surfactin may reduce the inhibition effect of fengycin against *R. stolonifer*.

Effects of fengycin with and without commercial surfactin added on spore germination in *R. stolonifer*

R. stolonifer spores were incubated in the PDB (potato dextrose broth) medium containing fengycin and spore generation was observed after incubation at 28°C for 6 h in a shaker at 100 rpm. Both the lipopeptide preparations affected spore germination in *R. stolonifer* (Tables 2). In the control group (the absence of lipopeptide), most spores had germinated, the spore balls had expanded and the germ tube had been formed. However, lipopeptide treatment was associated with a gradual decline in the germination of spores and the length of germ tube became shorter. Additionally, fengycin showed greater antifungal activity before commercial surfactin was added. Even when the concentration of fengycin was the same, treatment with the lipopeptide preparation without commercial surfactin added produced spores with shorter germ tube than treatment with the preparation with commercial surfactin added. The results indicated that commercial surfactin may decrease the inhibitory effect of fengycin on spore germina-

Table 2. The influence of fengycin with and without commercial surfactin added on spore germination in *R. stolonifer*

Fengycin concentration (μg/ml)	Fengycin without commercial surfactin added			Fengycin with commercial surfactin added		
	Germination (%)	Inhibition[a] (%)	Length of germ tube (μm)	Germination (%)	Inhibition[a] (%)	Length of germ tube (μm)
Control[b]	91±1	0A	75.5±0.57A	91±0	0A	80.1±0.45A
25	81±0	11.0B	64.4±0.69B	85±1	6.6B	77.5±0.66A
50	70±1	23.1C	50.7±0.54C	80±1	12.1C	68.6±0.58B
100	59±1	36.3D	39.6±0.31D	57±0	37.4D	45.3±0.34C
200	31±0	65.9E	19.9±0.56E	46±1	49.5E	37.8±0.43D
400	12±0	86.8F	7.8±0.45F	40±1	56.0F	20.6±0.27E
800	2±0	97.8G	-	23±0	74.7G	10.9±0.25F

[a] Inhibition values were calculated using the formula: Inhibition(%) = [1 - germination rate of treatment(%) / germination rate of control(%)] × 100
[b] Control indicates no lipopeptide treatment.
Different letters within columns indicate significant differences at P<0.05, according to Tukey's multiple range test.

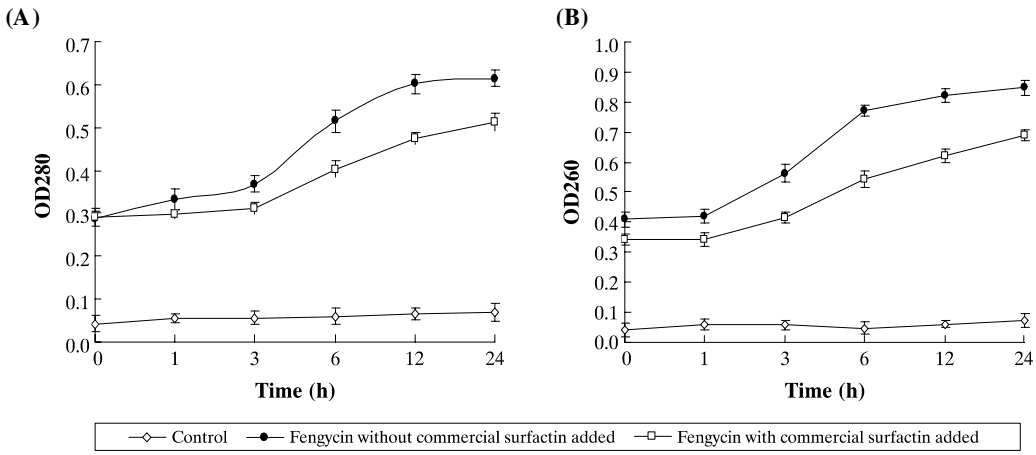

Fig. 1. Amount of protein and nucleic acid released by fengycin from *R. stolonifer* hyphae. (A) The amount of protein released from *R. stolonifer* hyphae; (B) The amount of nucleic acid released from *R. stolonifer* hyphae.

tion in *R. stolonifer*.

Measurement of protein and nucleic acid released from hyphae

Although the overall antibiotic mechanisms of antimicrobial peptides have not been clearly elucidated, disruption of the cell structure by pore formation (Kim *et al*., 2001) or ion channel generation seems to be the most likely mechanism (Bechinger *et al*., 1993). To investigate whether fengycin acts on the cell membrane and changes its permeability, the ultraviolet absorption due to substances such as protein and nucleic acid outside the cell were measured using a colorimetric

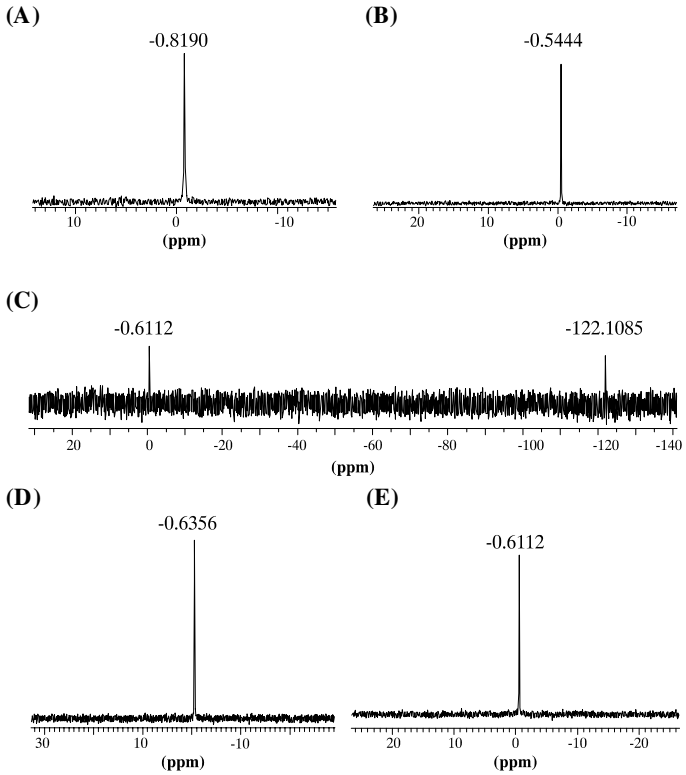

Fig. 2. ^{31}P NMR spectra of DPPC (1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine) dispersions. (A) control dispersions; (B) DPPC dispersions with DPPC: fengycin (no commercial surfactin added) 16:1; (C) DPPC dispersions with DPPC: fengycin (no commercial surfactin added) 8:1; (D) DPPC dispersions with DPPC: fengycin (commercial surfactin added) 16:1; (E) DPPC dispersions with DPPC: fengycin (commercial surfactin added) 8:1.

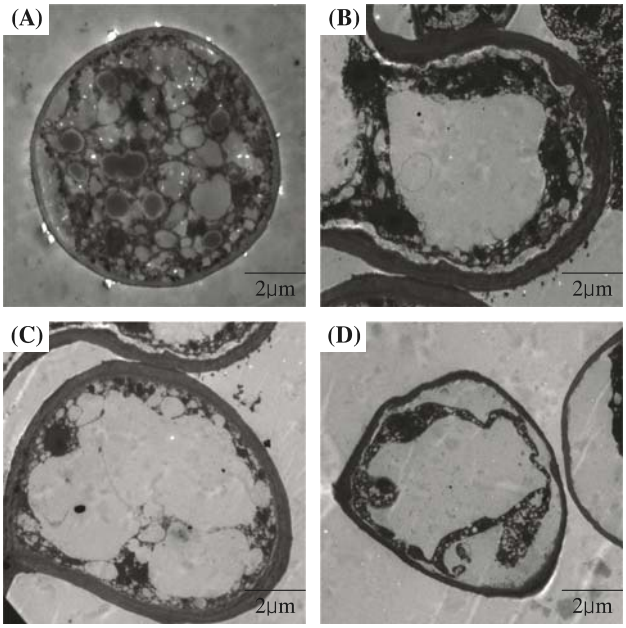

Fig. 3. TEM micrographs of *R. stolonifer* hyphae. (A) No lipopeptide-treated hyphae showing normal cell wall, nucleus, mitochondria and vacuole. (B) Fengycin-treated hyphae showing uneven cell wall. (C) Fengycin-treated hyphae showing large vacuoles inside the cells. (D) Fengycin-treated hyphae showing the cytoplasm separated from cell wall and cell membrane.

method. After being cultured for 2 days, the hyphae (4 g humid weight) were washed in isotonic NaCl, then incubated in 40 ml phosphate buffer (pH 7.2) containing fengycin (200 μg/ml) at 28°C for various periods of time. The OD_{260} and OD_{280} were measured using a Shimazu UV-2450 spectrophotometer (Japan). The result showed that both the two lipopeptide preparations in the presence and absence of commercial surfactin caused leakage of protein and nucleic acid from the hyphae of *R. stolonifer* (Fig. 1). This effect was observed after about 3 h of fengycin treatment. The increase in the amount of protein and nucleic acid released from the hyphae after fengycin treatment provides evidence that fengycin acts on the plasma membrane by forming pores which allow leakage of cell-substances from the cell. In this experiment, the leakage caused by fengycin without surfactin added was higher than that caused by fengycin with surfactin added, but the difference was insignificant.

Interaction of fengycin with and without commercial surfactin added in model membrane

Small artificial unilamellar vesicles (SUVs) were used to form model membrane systems. Model membranes were prepared by dissolving 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC 2 mg, Sigma) and fengycin (DPPC: fengycin 8:1 and 16:1, concentration ratio) in 600 μl of chloroform/methanol (2:1, v/v). The solution was dried under a stream of nitrogen gas and then vacuum pumped overnight. The dry powder was hydrated by addition of deuterium oxide. ^{31}P experiments were carried out using a FT-NMR spectrometer (BRUKER, AVANCE 500, Switzerland).

The ^{31}P NMR spectra of DPPC dispersion were shown in Fig. 2. In the dispersion of DPPC along, the chemical shift of phosphoryl group was -0.8190 ppm (Fig. 2A) and the chemical shift in the dispersion containing fengycin (no commercial surfactin added) in 16:1 DPPC/fengycin concentration ratio was changed to -0.5444 ppm (Fig. 2B). Furthermore, when the concentration ratio of DPPC and fengycin (no commercial surfactin added) was 8:1, two peaks could be seen in the spectrum as the structure of phosphoryl group had been damaged (Fig. 2C). In addition to the peak at around -0.6112 ppm, there was a new peak at around -122.1085 ppm. The ^{31}P NMR spectra indicated that fengycin had acted on the phosphoryl group of DPPC to disrupt the structure of the liposome.

The results we had studied indicated that the antifungal activity of fengycin decreased after commercial surfactin was added; the NMR spectra also showed that fengycin without commercial surfactin added acted on the liposome with greater effect than fengycin with commercial surfactin added. When the concentration ratio of DPPC and fengycin (commercial surfactin added) was 16:1 and 8:1, the chemical shift of phosphoryl group was -0.6356 and -0.6112 ppm, respectively (Figs. 2D and E). In the same DPPC/fengycin concentration ratio, the change in chemical shift of phosphoryl group was smaller in the presence of commercial surfactin. These results indicated that commercial surfactin may alleviate the interaction between fengycin and the model membrane.

TEM (transmission electron microscopy) observations on the hyphae of *R. stolonifer* treated with fengycin

The internal structure of the hyphae after fengycin treatment was observed with TEM (Hernández-Lauzardo *et al*., 2008). In TEM photographs, organelles such as mitochondria, nucleus and vacuole could be clearly seen in the untreated hyphae and the enclosing cell wall was well defined (Fig. 3A) while the cell wall of the lipopeptide-treated *R. stoloenifer* hyphae was uneven (Fig. 3B). There was a large vacuole inside the cells of the lipopeptide-treated hyphae (Fig. 3C) which was rarely seen in control cells. Furthermore, the cytoplasm was

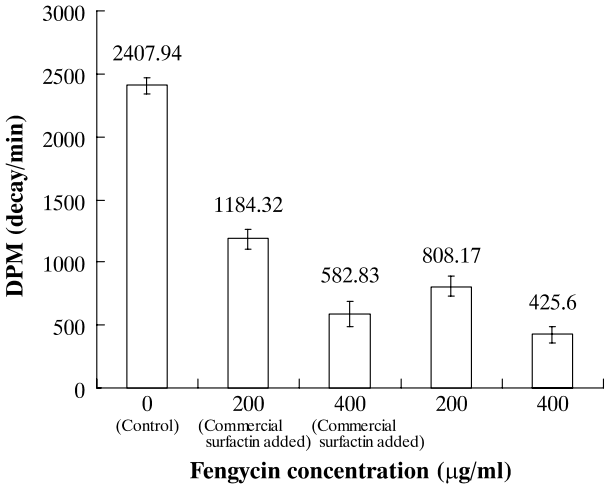

Fig. 4. Radioactivity of *R. stolonifer* hyphae after fengycin treatment.

separated from the cell wall and cell membrane (Fig. 3D). These results indicated that fengycin had penetrated the cell membrane and interacted with cellular organelles.

^{3}H-thymidine (^{3}H-TdR) incorporation assay

As the precursor of DNA synthesis, ^{3}H-TdR can be incorporated into the cell DNA specifically. Since tritium radiates β-ray with low energy and biotoxicity, it can be easily traced. The hyphae were incubated in the PDB medium containing fengycin and ^{3}H-TdR (1.85×10^{5} Bq/ml) for 24 h. The radioactivity of hyphae was measured using a liquid scintillation counter (BECKMAN, LS3801, USA).

The effect of fengycin and commercial surfactin on DNA synthesis of *R. stolonifer* was shown in Fig. 4. Compared to the radioactivity of the control haphae, fengycin treatment resulted in a decrease in the radioactivity of hyphae. These results indicated that fengycin prevented *R. stolonifer* from utilizing ^{3}H-TdR and inhibited the DNA synthesis after it reached the inner structure through cell membrane. Moreover, it was also found that the hyphae treated with the lipopeptide preparation with commercial surfactin added showed higher radioactivity than that treated without commercial surfactin added, indicating that they used more ^{3}H-TdR in the process of DNA synthesis and commercial surfactin may reduce the inhibition effect of fengycin against DNA synthesis of *R. stolonifer*.

In summary, fengycin produced by *B. subtilis* fmbJ has antifungal activity against the filamentous fungus, *R. stolonifer*. The fungal cell membrane is the prime site for antimicrobial attack by fengycin, although treatment with fengycin also leads to a series of other important changes in fungal cells. And meanwhile, commercial surfactin may reduce the antifungal activity of fengycin. Lastly, the results of our work may be of importance for a better and more reasonable use of the lipopeptide antibiotics.

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