Journal of Microbiology (2014) Vol. 52, No. 8, pp. 675–680
DOI 10.1007/s12275-014-3605-3

Effects of Fengycin from *Bacillus subtilis* fmbJ on Apoptosis and Necrosis in *Rhizopus stolonifer*

Qunyong Tang, Xiaomei Bie*, Zhaoxin Lu, Fengxia Lv, Yang Tao, and Xiaoxu Qu

College of Food Science and Technology, Nanjing Agricultural University, Key Laboratory of Food Processing and Quality Control, Ministry of Agriculture of China, P. R. China

(Received Nov 20, 2013 / Revised May 27, 2014 / Accepted May 28, 2014)

The lipopeptide antibiotic fengycin, produced by *Bacillus subtilis*, strongly inhibits growth of filamentous fungi. In this study, we evaluated the effects of fengycin treatment on apoptosis and necrosis in *Rhizopus stolonifer* by means of cell staining and epifluorescence microscopy. At fengycin concentrations less than 50 μg/ml, treated fungal cells demonstrated a dose-dependent increase in apoptosis-associated markers compared with the untreated control. These markers included chromatin condensation, reactive oxygen species accumulation, mitochondrial membrane potential depolarization, phosphatidylserine externalization, and the occurrence of DNA strand breaks. These results showed that fungal cells were impaired in a number of important functions and entered apoptosis upon treatment with low concentrations of fengycin. In contrast, high concentrations (>50 μg/ml) induced necrosis, indicating that the fungicidal action of fengycin operates via two modes: apoptosis at low concentrations and necrosis at high concentrations. Additionally, the apoptotic effect that we have shown suggests that lower concentrations of fengycin than previously thought may be effective for food preservation.

Keywords: apoptosis, food preservation, fengycin, *Bacillus subtilis*, *Rhizopus stolonifer*

Introduction

Soft rot disease, caused by the fungus *Rhizopus stolonifer,* is a significant problem in post-harvest storage of peaches and apples (Ogawa *et al.*, 1995; Fan and Tian, 2000). Synthetic fungicides are commonly used to control harvested fruit diseases (Eckert and Ogawa, 1988), but they also raise concerns for public health and the environment (Spotts and Cervantes, 1986). In recent years, biological control has become an attractive alternative for preventing fruit post-harvest diseases (Droby *et al.*, 1991; Chand and Spotts, 1996).

The bacterium *Bacillus subtilis* produces antifungal lipopeptides such as iturin, mycosubtilin, and fengycin; thus, fengycin can be used as an effective fungicide for agricultural biocontrol (Gueldner *et al.*, 1988; Asaka and Shoda, 1996). In our previous study, the fmbJ strain of *B. subtilis* was found to produce fengycin A and B (Fig. 1), which displayed significant inhibitory activity against the growth of *R. stolonifer* (Bie *et al.*, 2009). In a separate study, Tao Yang examined the antifungal mechanism of fengycin on *R. stolonifer*, finding that fengycin attacked the integrity and structure of the cell membrane, thus altering its permeability and eventually inducing necrotic cell death (Tao *et al.*, 2011). Notably, this mechanism was based on the use of relatively high concentrations of fengycin, and the effects of low concentrations were not studied.

Apoptosis is a core cellular process of eukaryotes, and it is recognized to play a key role in the development of animals at all stages of life, from birth to death. Surprisingly, the potential importance of apoptosis in the fungal life cycle has received scant attention and has remained largely unrecognized until recently (Madeo *et al.*, 1997; Roze and Linz, 1998; Qi *et al.*, 2010). Consequently, it is unknown whether the lipopeptide, fengycin, might influence apoptosis in fungi. Other antifungal peptides, such as PAF produced by *Penicillium chrysogenum* and surfactin produced by *B. subtilis*, were shown to elicit pores in cell membranes at high concentrations and to trigger apoptosis at low concentrations (Hagen *et al.*, 2007; Kim *et al.*, 2007). Furthermore, some non-peptidyl antifungal substances such as α-tomatine, osmotin, and killer toxin have also been found to cause cell death via either necrosis or apoptosis for high and low concentrations, respectively (Narasimhan *et al.*, 2001; Reiter *et al.*, 2005; Ito *et al.*, 2007).

Fengycin may act in a similar manner. Thus, the present study was designed to test the hypothesis that fengycin induces *R. stolonifer* cell death via two modes of action: necrosis at high concentrations and apoptosis at low concentrations.

Materials and Methods

Strains

Rhizopus stolonifer (AS 3.2336) was obtained from the Institute of Microbiology, Chinese Academy of Sciences (IMCAS). *Bacillus subtilis* fmbJ (CGMCC No.0943) is the wild-type strain isolated from soil and characterized in the laboratory as a producer of fengycin (Liang and Lu, 2001).

*For correspondence. E-mail: bxm43@njau.edu.cn; Tel.: +86-25-84396570; Fax: +86-25-84396431

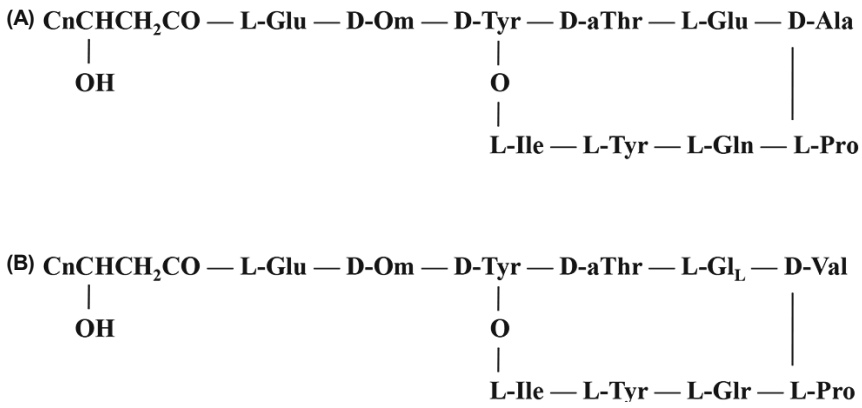

Fig. 1. **The chemical structure of fengycin A (A) and fengycin B (B).**

Fermentation and preparation of fengycin

Bacillus subtilis fmbJ was inoculated into a 250 ml shake flask containing 50 ml of beef extract medium (3 g/L beef extract, 10 g/L peptone, 5 g/L NaCl, pH adjusted to 7.2) and precultured at 37°C with shaking at 130 rpm for 24 h. A sample of this preculture (10 ml) was inoculated into a 1,000 ml shake flask containing 200 ml of Landy medium (20 g/L glucose, 5 g/L yeast extract, 5 g/L L-glutamic acid, 1.0 g/L KH_2PO_4, 0.16 mg/L $CuSO_4$, 0.5 g/L $MgSO_4$, 0.15 mg/L $FeSO_4$, 0.5 g/L KCl, 5.0 mg/L $MnSO_4{\cdot}H_2O$, pH adjusted to 7.0), then cultivated at 33°C with shaking at 180 rpm for 50 h. The culture broth was centrifuged at 11,000 × *g* for 15 min to remove bacterial cells. Fengycin was precipitated with 6 M HCl and extracted using methanol. The resulting supernatant was concentrated by vacuum freeze-drying and dissolved in sterile water.

High performance liquid chromatography (HPLC)

Fengycin extracts was analyzed via HPLC (reversed phase C18 analytical column; 250 mm × 4.6 mm; Agilent, USA) at a flow rate of 0.5 ml/min using gradient elution and a detection wavelength of 230 nm. Mobile phase A was water with 0.1% trifluoroacetic acid and mobile phase B was acetonitrile with 0.1% trifluoroacetic acid. The elution conditions were as follows: 0–80 min with 0–70% (v/v) A and 30–100% (v/v) B (Tao *et al.*, 2011).

Detection of apoptosis

Hoechst 33258 staining: *R. stolonifer* mycelium was treated with fengycin for 4 h, then stained with 0.1 μg/ml of Hoechst 33258 at 28°C for 5 min, and finally, observed under an epi-fluorescence microscope (Zeiss, Germany) using a [346 nm/460 nm] filter.

Detection of reactive oxygen species in fungal cells: Production of reactive oxygen species (ROS) by *R. stolonifer* was detected by monitoring the conversion of non-fluorescent dihydrorhodamine 123 (DHR123) to red fluorescent rhodamine 123 (Rh123). After treatment with fengycin for 6 h, *R. stolonifer* was incubated with 1% (w/v) snail enzyme and cellulase for 2 h, stained with 5 μg/ml DHR123 (Sigma-Aldrich, USA) for 30 min, and then observed by fluorescence microscopy using a [507 nm/529 nm] filter.

JC-1 staining: Mitochondrial membrane potential (ΔΨm) was assessed by using a JC-1 mitochondrial membrane po-

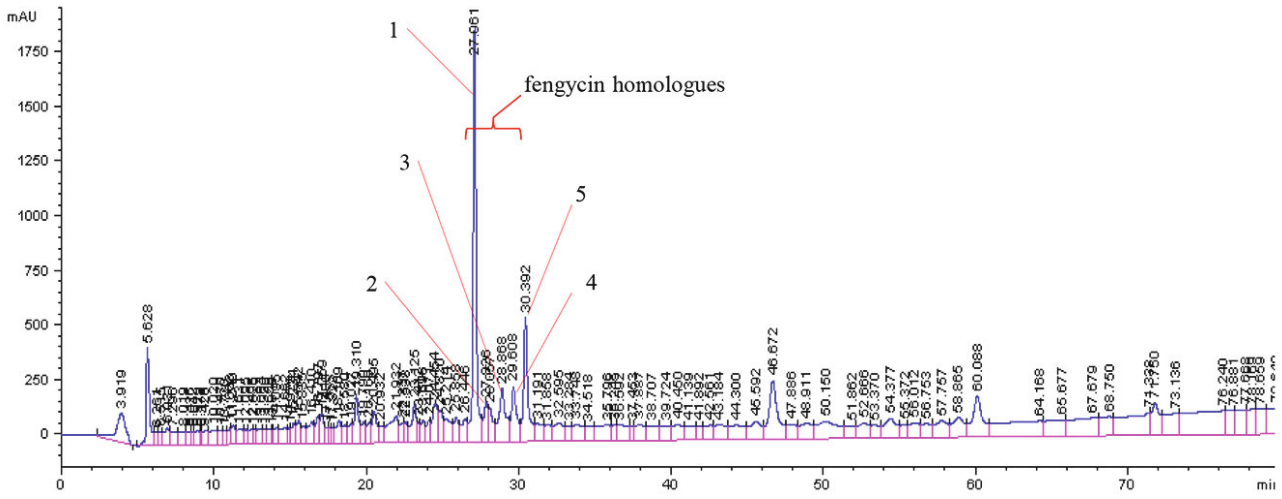

Fig. 2. **HPLC chromatograms of lipopeptides.** Homologues of fengycin were analyzed by an elution using a linear gradient of 0–70% (v/v) solvent A (water with 0.1% trifluoroacetic acid) and 30–100% solvent B (acetonitrile with 0.1% trifluoroacetic acid) over 80 min, at a flow rate of 0.5 ml/min.

tential assay kit (Beyotime, China), following the manufacturer's protocol. JC-1 can be used as an indicator of mitochondrial potential in a variety of cell types. The dye selectively enters mitochondria where it forms green fluorescent monomers at relatively low ΔΨm and red fluorescent J-aggregates at relatively high ΔΨm. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. After treatment with fengycin, the mycelium was incubated at 37°C for 20 min with 5 μg/ml JC-1, then washed twice with PBS and observed under the fluorescence microscope using a [515 nm/529 nm] filter.

Propidium iodide (PI) and annexin V staining: To examine cellular integrity and phosphatidylserine (PS) externalization, we stained the protoplasts of *R. stolonifer* with PI and FITC-conjugated annexin V using an Annexin V-FITC Apoptosis Detection kit (Beyotime). PI is a fluorochrome that cannot cross the membrane of living cells. However, PI can readily penetrate dead cells to stain DNA. After treatment with different concentrations of fengycin for 4 h, *R. stolonifer* was incubated with snail enzyme and cellulase as above, and then stained with FITC-conjugated annexin V at 28°C for 15 min, before microscopic observation of PS externalization in the fungal cells, using a [488 nm/525 nm] filter.

TUNEL assay: DNA strand breaks in fengycin-treated fungal cells were indicated by enzymatic (terminal deoxynucleotidyl transferase) labeling of free 3′-OH termini with FITC-conjugated deoxyuridine triphosphate (dUTP). After treatment with fengycin for 6 h, DNA strand breaks in *R. stolonifer* were detected using a commercial kit (Beyotime). Fungal mycelium was fixed with 4% (v/v) paraformaldehyde. Subsequently, mycelium was washed with phosphate buffered saline (PBS), resuspended in a permeabilization solution (0.1% [v/v] Triton X-100) for 2 min and incubated with the TUNEL reaction mixture. Mycelia were observed via fluorescence microscopy using a [488 nm/525 nm] filter.

Results

Determination of the fengycin

B. subtilis fmbJ was inoculated into Landy medium and cultivated at 33°C for 50 h. The fermentation broth after centrifugation was precipitated with 6 M HCl and extracted by methanol. After centrifugation at 10,000 × *g* for 15 min, the resulting fengycin solution was analyzed by reversed phase high performance liquid chromatography (Fig. 2). According to the mass spectrometry analysis, the retention time of fengycin homologues was between 24 min to 32 min, and the molecular weight of fengycin homologues had been identified previously as from 1435 Da to 1505 Da (Bie *et al.*, 2009). From the HPLC chromatograms, the concentration of fengycin was calculated using a standard curve, and the yield of fengycin was found to be 700 mg/L of fermentation broth.

Fengycin-induced chromatin condensation

Hoechst 33258 was used to evaluate the effects of fengycin treatment on the hyphal nuclei of *R. stolonifer*. These were stained with Hoechst 33258 in a dose-dependent manner

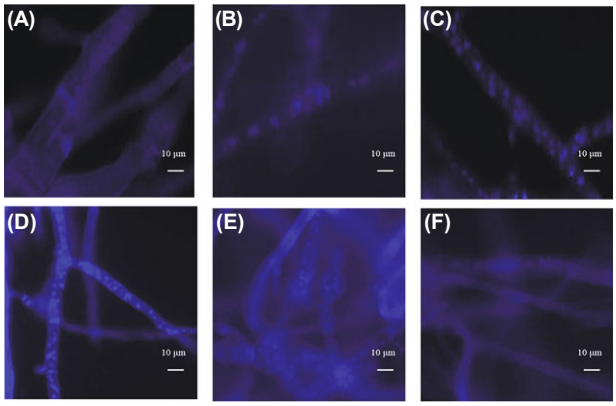

Fig. 3. Fengycin-induced chromatin condensation. Representative fluorescence micrographs of *R. stolonifer* hyphal cells stained with Hoechst 33258 after 4 h treatment with 0 μg/ml (A), 6.25 μg/ml (B), 12.5 μg/ml (C), 25 μg/ml (D), 50 μg/ml (E), or 100 μg/ml (F) fengycin. Magnification, 600×.

(Figs. 3C–3D), exhibiting low to high fluorescence at fengycin doses from 6.25 to 25 μg/ml, respectively. For all concentrations tested, staining levels were substantially higher than those of the untreated control (Fig. 3A). At a concentration of 12.5 μg/ml, half of the cells were stained by Hoechst 33258. Thus, this fengycin concentration may be the IC50 value for apoptosis of cells in *R. stolonifer*. When the concentration of fengycin reached 50 μg/ml, hyphal swelling and chromatin condensation became evident (Fig. 3E); these are typical markers of apoptosis. When fungal cells were exposed to fengycin at 100 μg/ml, the mycelial structure became blurred, which was regarded as indicative of cell necrosis (Fig. 3F).

Fengycin-induced reactive oxygen species accumulation

ROS accumulation was detected by monitoring the convertsion of non-fluorescent DHR123 to red fluorescent Rh123. In treated *R. stolonifer* cells, fluorescence increased in a dose-

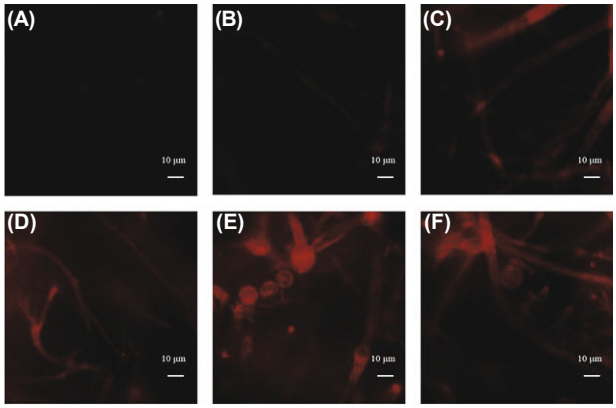

Fig. 4. Effect of fengycin on reactive oxygen species (ROS) accumulation. Representative fluorescence micrographs of *R. stolonifer* hyphal cells stained with dihydrorhodamine 123 after 4 h treatment with 0 μg/ml (A), 6.25 μg/ml (B), 12.5 μg/ml (C), 25 μg/ml (D), 50 μg/ml (E), or 100 μg/ml (F) fengycin. Magnification, 600×.

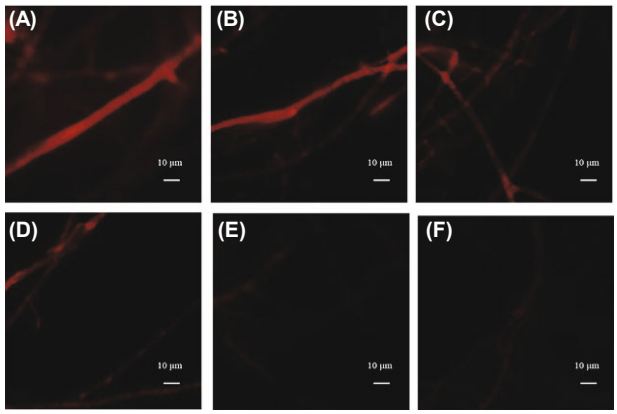

Fig. 5. Effect of fengycin on mitochondrial membrane potential. Representative fluorescence micrographs of *R. stolonifer* hyphal cells stained with JC-1 after 4 h treatment with 0 μg/ml (A), 6.25 μg/ml (B), 12.5 μg/ml (C), 25 μg/ml (D), 50 μg/ml (E), or 100 μg/ml (F) fengycin. Magnification, 600×.

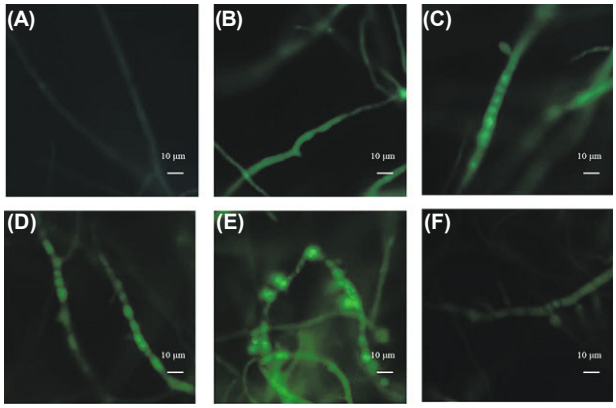

Fig. 7. Effect of fengycin on the occurrence of DNA strand breaks. Representative fluorescence micrographs of *R. stolonifer* hyphal cells subjected to a TUNEL assay after 4 h treatment with 0 μg/ml (A), 6.25 μg/ml (B), 12.5 μg/ml (C), 25 μg/ml (D), 50 μg/ml (E), or 100 μg/ml (F) fengycin. Magnification, 600×.

dependent manner, showing low to high fluorescence at doses from 6.25 to 25 μg/ml, respectively (Figs. 4B–4F). Almost no fluorescence was observed for the untreated control (Fig. 4A). As was the case for the Hoechst experiment, hyphae manifested swelling when fengycin concentrations reached 50 μg/ml (Fig. 4E). Hyphal swelling and ROS accumulation are consistent with the occurrence of apoptosis in the fengycin-treated cells.

Fengycin-induced depolarization of the mitochondrial membrane potential

As shown in Fig. 5A, the untreated hyphae of *R. stolonifer* exhibited a strong red fluorescence, indicating a high ΔΨm. Significantly, in treated hyphae the fluorescence became weaker with increasing fengycin dosage (Figs. 5B–5F), indicating that the mitochondria were depolarized by the treatment. At a concentration of 50 μg/ml the hyphal cells exhibited features typical of early-stage apoptosis (Fig. 5E), and at 100 μg/ml cell necrosis (almost no fluorescence) was observed (Fig. 5F). These results are consistent with the occurrence of apoptosis in fengycin-treated *R. stolonifer* cells.

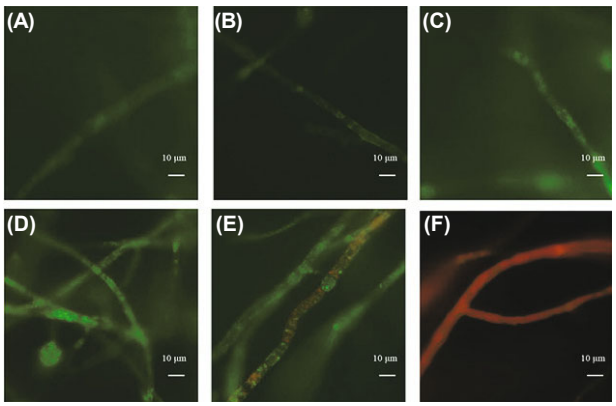

Fig. 6. Effect of fengycin on phosphatidylserine (PS) externalization and cell membrane permeability. Representative fluorescence micrographs of *R. stolonifer* hyphal cells co-stained with FITC-conjugated Annexin V (PS externalization; green) and propidium iodide (membrane permeability; red) after 4 h treatment with 0 μg/ml (A), 6.25 μg/ml (B), 12.5 μg/ml (C), 25 μg/ml (D), 50 μg/ml (E), or 100 μg/ml (F) fengycin. Magnification, 600×.

Effects of fengycin on PS externalization and cell membrane integrity

For the annexin V assay, we found that untreated control hyphae produced only weak green fluorescence (Fig. 6A). This was elevated after treatment with 6.25 μg/ml fengycin, and fluorescence continued to rise in a concentration-dependent manner up to 50 μg/ml; this indicates dose-dependent induction of phosphatidylserine externalization and hence, apoptosis (Figs. 6B–6D). PI staining (red fluorescence) was not observed for concentrations less than 50 μg/ml (Figs. 6A–6D), but partial staining became apparent at 50 μg/ml, (Fig. 6E), indicating the onset of membrane damage. At 100 μg/ml fengycin, the whole mycelium exhibited strong red fluorescence (Fig. 6F), showing that PI could readily penetrate the membranes of the now necrotic cells.

Fengycin-induced DNA strand breaks (apoptosis)

In untreated control hyphae we observed only a weak green fluorescence (Fig. 7A). Fluorescence intensity was greater for all fengycin-treated groups and it increased with concentration from 6.25 to 50 μg/ml (Figs. 7B–7E); this indicates the occurrence of DNA fragmentation due to activation of endonucleases, which is typical of apoptotic cells. In contrast, at the high concentration of 100 μg/ml, fluorescence was nearly as low as the control (Fig. 7F).

Discussion

The main objectives of this study were to investigate whether fengycin can induce apoptosis in *R. stolonifer* and whether its fungicidal effect is bi-modal; operating via necrosis or

apoptosis at high and low concentrations, respectively.

ROS play important roles in the induction of apoptosis under both physiological and pathological conditions. ROS accumulation is considered to be essential for apoptosis in some species and to be a crucial signal for downstream apoptotic events (Masato *et al.*, 1998; Robson, 2006). In the present study, fengycin was shown to increase ROS levels in accordance with dose. In a previous study, we showed that the activity of mitochondrial NADH dehydrogenase was reduced to 50% by 25 μg/ml fengycin *in vivo* (Tang *et al.*, 2011). Here, JC-1 staining showed that the mitochondria were depolarized after treatment with fengycin. These results indicate that, at lower concentrations (<50 μg/ml), fengycin induced the mitochondrial dysfunction and ROS accumulation that is typical of apoptotic cells. On the other hand, above this concentration, necrotic cell lysis occurred as demonstrated by a blurring of structures seen under the microscope that was due to the break-up of the cells.

In order to further elucidate the effect of fengycin on apoptosis in *R. stolonifer*, we examined PS externalization and membrane integrity via annexin V and PI staining, respectively. The results revealed that, at low concentrations (<50 μg/ml), PI fluorescence was essentially absent while PS externalization increased in a dose-dependent manner; this indicates that cells underwent apoptosis rather than necrosis after treatment with fengycin. However, when the fungal cells were exposed to fengycin at 50 μg/ml, partial PI staining was observed, and at 100 μg/ml, PI completely permeated the mycelium, showing that necrosis dominated at high concentrations.

Our results demonstrated that nuclear alterations in the form of condensed chromatin (Fig. 3) and DNA strand breaks (Fig. 7) were induced by fengycin at lower concentrations. The data from the TUNEL assay indicated that the DNA strand breaks increased with the rising dose of fengycin. However, this correlation was reversed when fengycin reached 100 μg/ml, which can be accounted for by the loss of endodeoxyribonucleases from necrotic cells. These results therefore lend further support to our hypothesis that low concentrations of fengycin trigger apoptosis, whereas high concentrations produce necrosis.

Conclusion

Our study examined a series of standard yet diverse apoptotic markers such as ROS accumulation, mitochondrial depolarization, phosphatidylserine externalization, chromatin condensation, and induction of DNA strand breaks. Based on these assays, we have shown that low concentrations (<50 μg/ml) of fengycin from *B. subtilis* fmbJ induced apoptosis in *R. stolonifer*. In contrast, higher concentrations induced necrosis, as indicated by gross structural defects, loss of membrane integrity, and absence of apoptosis-associated DNA strand breaks. In addition, these results indicate that fengycin may be effective for food preservation at lower concentrations than previously thought, and this could reduce potential health and environmental concerns associated with its prophylactic use.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (Nos. 30671460 and 30871753).

References

Asaka, O. and Shoda, M. 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. *Appl. Environ. Microbiol.* **62**, 4081–4085.

Bie, X.M., Lu, Z.X., and Lv, F.X. 2009. Identification of fengycin homologues from *Bacillus subtilis* with ESI-MS/CID. *J. Microbiol. Methods* **79**, 272–278.

Chand, G.T. and Spotts, R.A. 1996. Postharvest biological control of blue mold of apple and brown rot of sweet cherry by natural saprophytic yeasts alone or in combination with low doses of fungicide. *Biol. Control.* **6**, 253–259.

Droby, S., Chalutz, E., and Wilson, C.L. 1991. Antagonistic microorganisms as biological control agents of postharvest diseases of fruits and vegetables. *Post-Harvest News Information* **2**, 169–173.

Eckert, J.W. and Ogawa, J.M. 1988. The chemical control of postharvest diseases: deciduous fruits, berries, vegetables, and root/tuber crops. *Annu. Rev. Phytopathol.* **26**, 433–469.

Fan, Q. and Tian, S.P. 2000. Postharvest biological control of Rhizopus rot of nectarine fruits by *Pichia membranefaciens*. *Plant Dis.* **84**, 1212–1216.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendal, R.F., Cox., R.H., Himmelsbach, D.S., Crumley, F.G., and Cutler, H.G. 1988. Isolation and identication of iturines as antifungal peptides in biological control of peach-brown rot with *Bacillus subtilis*. *J. Agr. Food Chem.* **36**, 366–370.

Hagen, S., Marx, F., Ram, A.F., and Meyer, V. 2007. The antifungal protein AFP from *Aspergillus giganteus* inhibits chitin synthesis in sensitive fungi. *Appl. Environ. Microbiol.* **73**, 2128–2134.

Ito, S., Ihara, T., Tamura, H., Tanaka, S., Ikeda, T., Kajihara, H., Dissanayake, C., Abddl, F.F., and Ei, M.A. 2007. α-Tomatine, the major saponin in tomato, induces programmed cell death mediated by reactive oxygen species in the fungal pathogen *Fusarium oxysporum*. *FEBS Lett.* **581**, 3217–3222.

Kim, S.Y., Kim, J.Y., Kim, S.H., Bae, H.J., Yi, H., Yoon, S.H., Koo, B.S., Kwon, M., Cho, J.Y., Lee, C.E., and *et al.* 2007. Surfactin from *Bacillus subtilis* displays anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signaling suppression. *FEBS Lett.* **581**, 865–871.

Liang, S.Y. and Lu, Z.X. 2001. The isolation, screening and identification of *Bacillus subtilis* strain producing high activity fibrinolysin. *Lett. Microbiol. China* **28**, 25–28.

Madeo, F., Frohlich, E., and Frohlich, K.U. 1997. A yeast mutant showing diagnostic markers of early and late apoptosis. *J. Cell Biol.* **139**, 729–734.

Masato, E., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. 1998. A caspase activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391**, 43–50.

Narasimhan, M.L., Damsz, B., Coca, M.A., Ibeas, J.I., Yun, D.J., Pardo, J.M., Hasegawa, M.H., and Bressan, R.A. 2001. A plant defense response effector induces microbial apoptosis. *Mol. Cell.* **8**, 921–930.

Ogawa, J.M., Zehr, E.I., Bird, G.W., Ritchie, D.F., Uriu, K., and Uyemoto, J.K. 1995. Compendium of Stone Fruit Diseases. pp. 98. APS Press.

Qi, G.F., Zhu, F.Y., Du, P., Yang, X.F., Qiu, D.W., Yu, Z.N., Chen, J.Y., and Zhao, X.Y. 2010. Lipopeptide induces apoptosis in fungal cells by a mitochondria-dependent pathway. *Peptides* **31**,

1978–1986.

Reiter, J., Herker, E., Madeo, F., and Schmitt, M.J. 2005. Viral killer toxins induce caspase-mediated apoptosis in yeast. *J. Cell Biol.* **168**, 353–358.

Robson, D. 2006. Programmed cell death in the *Aspergilli* and other filamentous fungi. *Med. Mycol.* **44**, 109–114.

Roze, L.V. and Linz, J.E. 1998. Lovastatin triggers an apoptosis-like cell death process in the fungus *Mucor racemosus*. *Fungal Genet. Biol.* **25**, 119–133.

Spotts, R.A. and Cervantes, L.A. 1986. Population, pathogenicity, and benomyl resistance of *Botrytis* spp., *Penicillium* spp., and *Mucor piriformis* in Packinghouses. *Plant Dis.* **70**, 106–108.

Tang, Q.Y., Zhou, X.H., Lu, Z.X., Lv, F.X., Wang, Y.F., and Bie, X.M. 2011. Effects of fengycin from *Bacillus subtilis* fmbJ on respiratory chain and nutrients utilization of *Rhizopus stolonifer*. *Food Sci.* **32**, 248–254.

Tao, Y., Bie, X.M., Lv, F.X., and Lu, Z.X. 2011. Antifungal activity and mechanism of fengycin in the presence and absence of commercial surfactin against *Rhizopus stolonifer*. *J. Microbiol.* **49**, 146–150.