

Characterization of *Fusarium graminearum* inhibitory lipopeptide from *Bacillus subtilis* IB

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Abstract *Bacillus subtilis* strain IB exhibiting inhibitory activity against the *Fusarium* head blight disease fungus *Fusarium graminearum* was isolated and identified. The major inhibitory compound was purified from the culture broth through anion exchange, hydrophobic interaction, and reverse phase high-performance liquid chromatography (RP-HPLC) steps. It was a 1,463-Da lipopeptide and had an amino acid composition consisting of Ala, Glx, Ile, Orn, Pro, Thr, and Tyr at a molar ratio of 1:3:1:1:1:1:2. Electrospray ionization mass spectrometry/mass spectrometry (ESI MS/MS) analyses of the natural and the ring-opened peptides showed the antagonist was fengycin, a kind of macrolactone molecule with antifungal activity produced by several *Bacillus* strains. Fluorescence microscopic analysis indicated this peptide permeabilized and disrupted *F. graminearum* hyphae.

Keywords *Bacillus subtilis* · Fengycin · *Fusarium graminearum* · *Fusarium* head blight disease

Introduction

Fusarium head blight (FHB) is a devastating disease that results in extensive reduction in the production and quality of wheat and barley throughout the world. *Fusarium graminearum* (sexual state, *Gibberella zeae*) is one of the principal pathogens of this disease and can infect wheat florets from the time of anthesis to the soft dough stage of

kernel development. Visible damages can be observed within 3–4 days if the environmental conditions are favorable for the development of this pathogen. Infected seeds show reduced germination rate and seedling vigor. *F. graminearum* also produces mycotoxins during the colonization of grain, and in some cases, during storage. Grains contaminated by mycotoxins are unsuitable for human consumption because they may cause numerous biological disturbances (Argyris et al. 2003; Goswami and Kistler 2004).

Application of fungicides is effective to control FHB caused by *F. graminearum*. But it inevitably leads to environmental pollution and drug tolerance of microbes. Biological control of *F. graminearum* has shown promise in previous studies (Fernandez 1992; Bujold et al. 2001; Khan et al. 2004). *Bacillus subtilis* strains are a rich source of antifungal peptides. The lipopeptides surfactins (Jitendra and Ibrahim 1997), the iturin family (Regine and Peypoux 1994), and fengycins (Vanittanakom and Loeffler 1986) are a prominent class of such compounds. These antibiotics are either cyclopeptides (iturins) or macrolactones (fengycins and surfactins) characterized by the presence of L and D amino acids and variable hydrophobic tails. These three kinds of lipopeptides are nonribosomal biosynthesized peptides. Large multienzymes catalyze all necessary steps in peptide biosynthesis, including the selection and ordered condensation of amino acid residues (Stein 2005). The productions of these three antifungal peptides play an important role in the biocontrol activity against a variety of plant pathogens of different *B. subtilis* strains. However, interest was mostly focused on iturin- or surfactin-producing *Bacillus* strains (Klich et al. 1994; Yu et al. 2002; Ahn et al. 2003; Cho et al. 2003; Bais et al. 2004; Kim et al. 2004). Fengycin consists of a β -hydroxy fatty acid connected to the N terminus of a decapeptide, including four D-amino acid residues and the rare amino acid L-

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omithine. The C-terminal residue of the peptide moiety is linked to the tyrosine residue at position 3, forming the branching point of the acylpeptide and the eight-membered cyclic lactone. Fengycin homologs are divided into two types (fengycins A and B) based on their amino acid sequence. If the amino acid at position 6 is Ala, it is fengycin A, and Val for fengycin B. The length of β -hydroxy fatty acid tail is variable (from C₁₄ to C₁₈) and links the amino group of its N-terminal amino acid Glu. Fengycin family shows potent antimicrobial activity, especially against filamentous fungi (Loeffler et al. 1986; Vanittanakom and Loeffler 1986; Vater et al. 2002; Williams et al. 2002). In this study, we isolated a *B. subtilis* strain with significant inhibitory activity against phytopathogen *F. graminearum*. The major inhibitory compound was structurally characterized as fengycin A by mass spectrometry and its effect on *F. graminearum* was studied with fluorescence microscope.

Materials and methods

Bacterial strain and culturing conditions

The *B. subtilis* strain IB was isolated from soil and identified by the Institute of Microbiology (Chinese Academy of Sciences). For production of the inhibitory compounds, a loop of IB cells from fresh slant culture was seeded into a 300-ml flask containing 20 ml medium (glycerol 10 g, peptone 20 g, MgSO₄ 1.5 g, K₂HPO₄ 1.5 g per liter, pH 7.0) and cultivated on a rotary shaker at 30°C and 180 rpm for 12 h. This seed culture was inoculated into a 500-ml flask containing 200 ml production medium [glucose (20 g), peptone (20 g), yeast extract (5 g), NaCl (5 g), K₂HPO₄ (1.5 g), MgSO₄ (1.5 g) per liter (pH 7.0)] and cultivated at the same condition for 60 h.

Fungus isolation and antifungal assay

F. graminearum was kept on PDA slants. The 4-day-old mycelia cultured on slants were spread on PDA plates, with 200- μ l samples from each purification step being loaded on the plates by stainless steel columns. The plates were incubated at 28°C for 4 days to detect the fractions containing inhibitory compounds. An inhibition zone would appear around the column containing inhibitory substances.

Purification of inhibitory compounds

One thousand milliliters of culture broth of IB was centrifuged at 9,000 rpm for 30 min and concentrated to 70 ml by a Millipore ultrafiltration apparatus with membranes cut-off molecules smaller than 3,000 Da. After being dialyzed against

buffer A (20 mM Na₂HPO₄–NaH₂PO₄, pH 6.5), the concentrated culture was loaded on a DEAE-Sepharose Fast Flow anion exchange column preequilibrated with buffer A. Buffer B [20 mM Na₂HPO₄–NaH₂PO₄ 1 M (NH₄)₂SO₄, pH 6.5] was used to elute the active fraction. This fraction was directly loaded on a SOURCE 15 PHE hydrophobic interaction column preequilibrated with buffer B. Most contaminants were washed with buffer A and the inhibitory compounds were finally eluted by Milli Q water. Further purification was performed on a C₁₈ reverse phase column (Dikma). A linear gradient solvent of 30% acetonitrile and 0.1% TFA to 80% acetonitrile and 0.1% TFA in 80 min was used to separate the inhibitory compounds.

Analytical methods

Amino acid composition analysis of the purified compound was done on an AminoQuant system after 20 mg of purified sample was hydrolyzed with 6 M HCl in vapor phase under an argon atmosphere for 24 h at 110°C.

To determine the lactone linkage, 1 mg of the lyophilized inhibitory compound was dissolved in 1 M potassium hydroxide (KOH) solution and allowed to react overnight at room temperature. Excess KOH was neutralized and the hydrolysate was desalted for mass spectrometry analysis.

All mass spectrometry analyses were done on a LCQ Advantage instrument with an electrospray ion source of Thermo Finnigan. Samples were infused with a syringe. The electrospray source was operated at a capillary voltage of 32 V, a spray voltage of 5 kV, and a capillary temperature of 320°C. For the collision-induced dissociation (CID) experiments, helium was used as the collision gas.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 15% polyacrylamide gels according to the protocol described by Laemmli (1970) and stained with Coomassie brilliant blue R-250.

The fluorescence reagents fluorescein diacetate (FDA, Molecular Probes) and propidium iodide (PI; Molecular Probes) were applied to assess the viability of *F. graminearum* hyphae. Fresh *F. graminearum* hyphae were incubated for 2 h at 28°C in the presence (50 μ g/ml) or absence of the inhibitory compound. Then the treated and control samples were stained with FDA and PI at a final concentration of 10 μ M for 25 min in the dark. After being washed with phosphate-buffered saline, the stained samples were immediately analyzed with fluorescence microscope.

Results

Isolation of *B. subtilis* IB

The antagonistic bacterium we named IB showed inhibitory activity against *F. graminearum* (Fig. 1). Isolate IB was

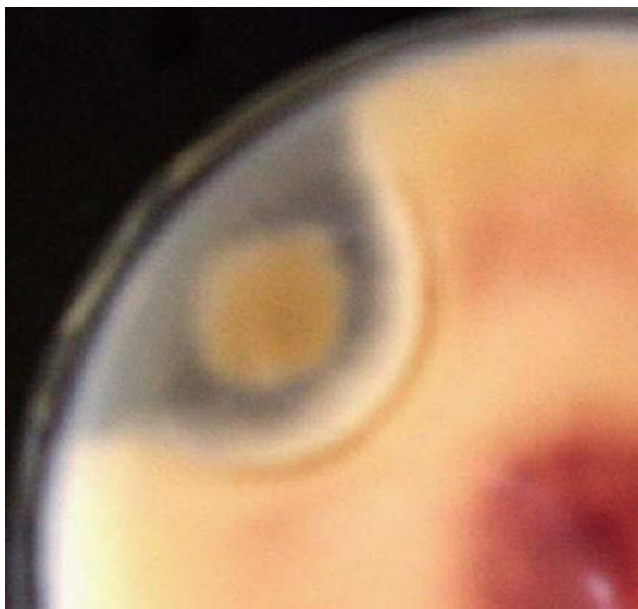


Fig. 1 Inhibition of *B. subtilis* IB against *F. graminearum* in PDA agar plate

identified according to *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbens 1984) by the Institute of Microbiology, Chinese Academy of Sciences. This microorganism was a motile gram-positive, spore-producing rod with a diameter smaller than 1 μm . It was able to grow at pH 5.7 or in 7% NaCl, but was unable to grow under anaerobic condition or at 50°C. IB was catalase, oxidase, Voges–Proskauer, starch hydrolysis, gelatin liquefaction, casein hydrolysis, nitrate reduction, and citrate utilization positive while its methyl red test was negative. IB was able to produce acid from glucose, xylose, arabinose, and mannitol, respectively, while it was unable to produce gas from glucose. Based on these morphological and physiological characteristics, IB was determined as a *B. subtilis* strain.

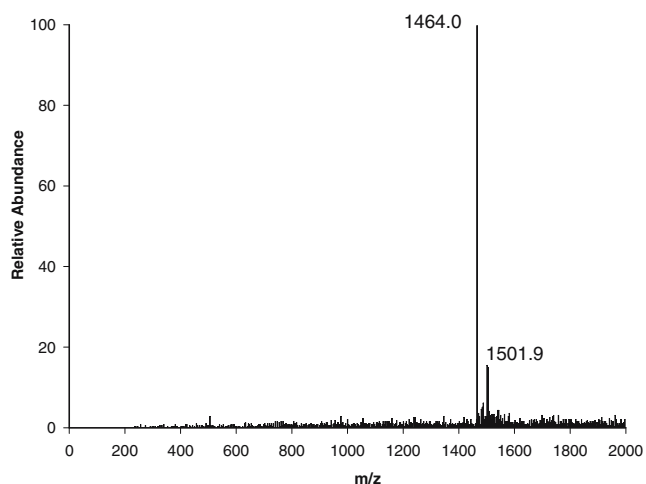


Fig. 2 ESI MS of the major active component. The $(\text{M}+\text{H})^+$ ion peak is at m/z 1464.0 and peak at m/z 1501.9 is $(\text{M}+\text{K})^+$

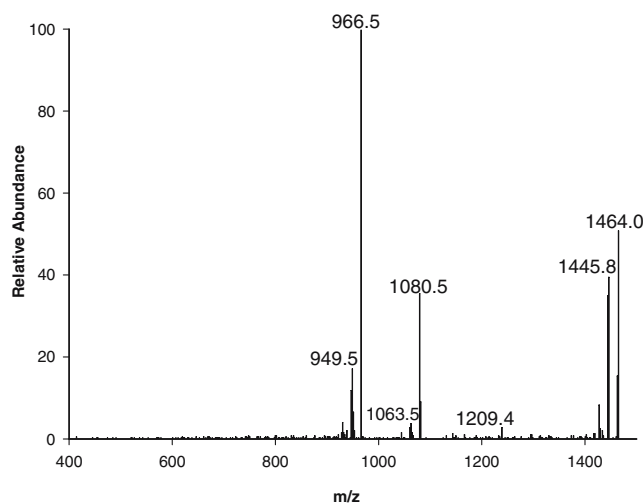


Fig. 3 CID analysis of the ion of m/z 1464

Purification of the major inhibitory compound

The compounds responsible for the inhibitory activity bound so strongly to the matrix of the hydrophobic interaction chromatography column that pure water was required to elute them. The active fraction collected in this step was analyzed by SDS-PAGE and displayed as a single band of about 7,000 Da. However, the electrospray ionization mass spectrum (ESI MS) of this fraction presented a cluster of peaks with 14 Da difference in their molecular weights (data not shown), indicating these molecules were homologs. Further purification by reverse phase high-performance liquid chromatography (RP-HPLC) was performed. From 1 l culture broth of IB, about 28 mg lyophilized powder of the homologs of the inhibitory compounds was collected and after the final purification step, 8 mg lyophilized powder of the major inhibitory compound was obtained. Besides *F. graminearum*, the major inhibitory compound also showed strong inhibitory activity against several other pathogenic fungi, including *Rhizoctonia solani*, *Botrytis cinerea*, and *Aspergillus nigers*. By ESI MS, the molecular weight of the purified inhibitory compound was determined to be 1,463 Da, which presented as protonated molecule (m/z 1464) and K^+ -adducted molecule (m/z 1501.9) (Fig. 2).

Amino acid composition analysis of the compound

The amino acid composition of this component was determined after using 6 M HCl to hydrolyze it. The result showed the compound consisted of Ala, Glx, Ile, Orn, Pro, Thr, and Tyr in a molar ratio of nearly 1:3:1:1:1:1:2. Glx represents Glu or Gln because they cannot be discriminated using this method. The nominal mass of a peptide only composed of these amino acid residues cannot be 1,463 Da, so it can be concluded that there is modification group in

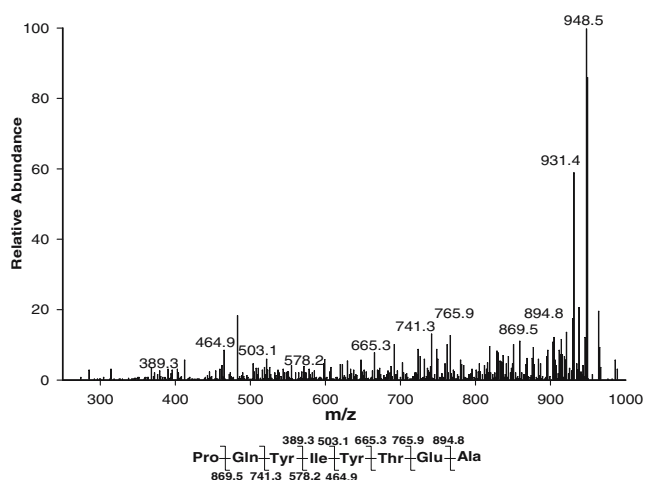


Fig. 4 CID analysis of the ion of m/z 966 and the deduced sequence

this peptide. An Edman degradation sequencing procedure was then run on this peptide, but no degradation was observed, indicating the N-terminal of this peptide was blocked.

MS analysis of the compound

To determine the structure of this peptide, ESI CID analysis of the natural molecule was done (Fig. 3). Based on the spectrum and the amino acid composition of the peptide, we could conclude ion of m/z 1209.4 represented the loss of the modification group at the N-terminal. m/z 1080.5 and 966.5 corresponded to the neutral losses of (side-chain Glu) and (side-chain Glu–Orn], respectively. Ion of m/z 1445.8 was responsible for the loss of one H_2O (18 Da) from m/z 1464, ions of m/z 1063.5 and 949.5 represented losses of one NH_3 (17 Da) from m/z 1080.5 and 966.5. Few ions were detected in the lower-mass region of the spectrum. Because the protonated ion of a peptide segment composed of the other eight amino acid residues will be of m/z at around 966, a second CID was performed using m/z 966.5 as the precursor ion. According to the nomenclature proposed by Roepstorff and Fohlman (1984), a series of *b*- and *y*-type ions were detected and a peptide sequence directed by Pro was deduced from this CID spectrum as Pro–Gln–Tyr–Ile–Tyr–Thr–Glu–Ala (Fig. 4). But the question arose that if the peptide segment composed of these eight amino acids had a free C-terminal, the protonated ion should be of m/z at about 983 (the difference between $-COOH$ and $-C=O$ is 17 Da). So it is possible that there is a cyclization involving the $-COOH$ group of the C-terminal amino acid. The $-OH$ group of Tyr or Thr may react with $-COOH$ and form a lactone bond. At this point, alkaline hydrolysis was done on the purified peptide. ESI MS of the hydrolysate showed a protonated peak of m/z 1482 was produced, the mass gain of 18 Da could be assigned to hydrolysis of a lactone. This

was similar to the result described by Williams et al. (2002) and indicated the inhibitory peptide did contain a cyclic domain. The hydrolyzed peptide was also sequenced by CID. From the product ions, the ring-opened peptide sequence was determined as Glu–Orn–Tyr–Thr–Glu–Ala–Pro–Gln–Tyr–Ile (Fig. 5). The sequence (Tyr–Thr–Glu–Ala) appeared in both the natural and the ring-opened peptide. Based on the results of CID analyses, the position of the lactone bond was determined: It linked the Tyr at position 3 and Ile. According to the above results, the structure of the purified inhibitory peptide produced by IB can be summarized as: side-chain Glu–Orn–Tyr–Thr–Glu–Ala–Pro–Gln–Tyr–Ile with a lactone bond linking the Tyr3 and Ile10. This structure is identical to that of C_{16} fengycin A (Williams et al. 2002).

Effect of the compound on *F. graminearum*

F. graminearum hyphae could be stained with FDA but not PI in the absence of fengycin A (Fig. 6a). In contrast, the treated hyphae could not be stained with FDA, whereas they were stained with PI (Fig. 6b). This indicated fengycin A permeabilized the membrane of *F. graminearum* hyphae. Viability test of *F. graminearum* in PDA media containing different concentrations of the purified lipopeptide showed the pathogen was unable to grow in the existence of 8 $\mu\text{g}/\text{ml}$ of the inhibitory compound.

Discussion

FHB disease of wheat and barley along with contamination of grains with mycotoxins attributable to the disease has spurred research on the fungal causal agent. As the principal pathogenic fungus, *F. graminearum* quickly has

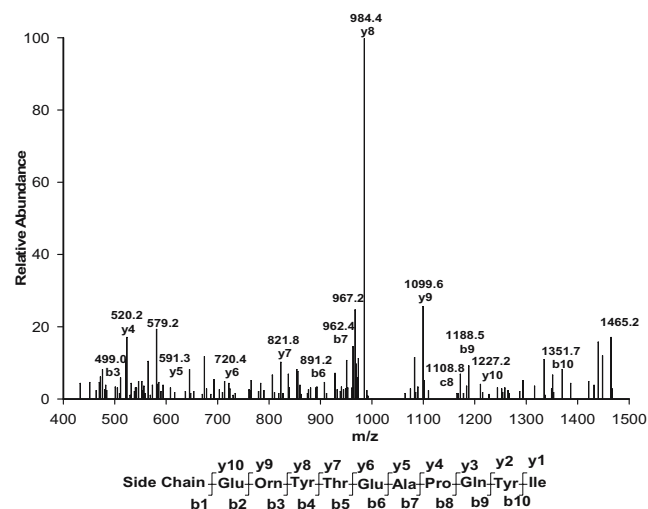
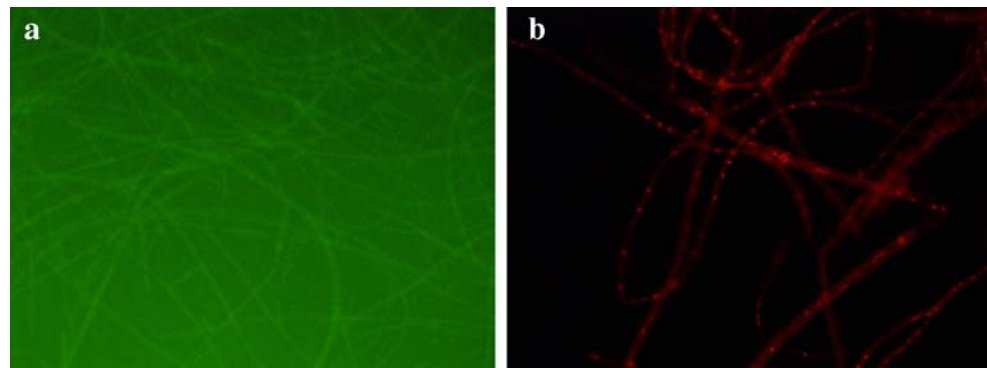


Fig. 5 CID analysis of the alkaline hydrolyzed peptide and the deduced sequence

Fig. 6 Fluorescent microscopy of *F. graminearum*. **a** Absence of fengycin A. Hyphae were stained by FDA and presented green fluorescence. **b** Presence of fengycin A. Hyphae were stained by PI and presented red fluorescence



become one of the most intensively studied fungal plant pathogens. Environmental pollution caused by excessive use and misuse of chemical pesticides has led to considerable changes in people's attitudes toward the use of pesticides in agriculture. So biological control of *F. graminearum* using antagonistic microorganisms may become an important way to reduce the damages brought about this pathogen (Han and Cao 2003).

B. subtilis IB with strong inhibitory activity against *F. graminearum* was isolated and the major inhibitory compound was determined as C₁₆ fengycin A. Although the reports of Toure et al. (2004) indicated fengycin homologs might be involved in the antagonistic activity of *B. subtilis* GA1 against *F. graminearum*, they detected the mixture of iturin, surfactin, and fengycin in the metabolites of GA1, so cannot exclude the possibility that the synergic effect of these three inhibitory lipopeptides suppressed the growth of *F. graminearum*. To our knowledge, this work was the first report that purified fengycin homolog inhibited the growth of *F. graminearum*. During our work, we also found C₁₆ fengycin A could suppressed the growth of *R. solani*, *B. cinerea*, and *A. nigers*, this confirmed the result of Hu et al. (2007) with fengycins-producing *B. subtilis* B-FS01 as antagonist against these fungi.

Fluorescein diacetate (FDA) is a kind of enzyme activity probe. Once inside cell membrane, it is cleaved by nonspecific esterase to release fluorescent group, which is inside the cell. Thus cell viability can be assessed by the accumulation of fluorescence. PI is a kind of nucleic acid probe. It is supposed to cross only through damaged membrane in cells. By using fluorescent probes, Chitarra et al. (2003) found iturin-like compounds produced by *B. subtilis* YM-10-20 permeabilized *Penicillium roqueforti* spores and blocked their germination. Kim et al. (2004) reported that a kind of fengycin-like lipopeptide secreted by *Bacillus thuringiensis* CMB26 inhibited *Colletotrichum gloeosporioides* by causing morphological changes. Until now there was no report on the effect by which fengycin homologs inhibited *F. graminearum*. In this study, by using fluorescent staining method, we gave a direct evidence that fengycin A suppressed the growth of *F. graminearum*

through the mechanism of destroying the membrane integrity of this pathogen and therefore, the metabolism of *F. graminearum* might be disturbed and its growth was inhibited. The MICs of fengycin against *Fusarium* sp. Tu 638 and *R. solani* Tu 8104 were 10 and 3.16 $\mu\text{g/ml}$, respectively (Vanittanakom and Loeffler 1986). As to iturin D and E, their MICs to *Fusarium oxysporum* were 30 and 40 $\mu\text{g/ml}$ (Besson and Michel 1987). We showed C₁₆ fengycin A was able to inhibit the growth of *F. graminearum* at the concentration of 8 $\mu\text{g/ml}$. These results showed that different fungi exhibited various sensitivities to antimicrobial lipopeptides, which might be explained by the difference of respective cell membranes of fungal strains.

Tandem mass spectrometry has been successfully used for sequencing peptide from different sources (Biemann 1995). Under CID, peptides fragment mostly along the peptide backbone and sequence information can be obtained because of different competitive cleavages along the peptide chain. For cyclic peptides, complexes may arise because ring opening occurs by cleavage of the *N*-acyl bonds in many places on the cyclic peptide backbone, leading to a mixture of different protonated linear peptide ions all with the same mass. However, if there is a proline in the cyclic peptide, the predominant ring-opened species will be a series of acylium ions resulting from cleavage of *N*-acyl bond at the *N*-terminal of the proline (Eckart 1994). Nishikiori et al. (1986) studied the structure of fengycin by spectrometry and chemical degradation and they localized the lactone linkage in fengycin between Tyr 3 and Ile 10 by chemical modification and NMR. In our work, proline directed fragmentation under CID mode was used to sequence the peptide of the cyclic domain of fengycin. Combining the peptide sequence information obtained from the ring-opened molecule by mild alkaline hydrolysis, we determined the Tyr 3→Ile 10 lactone linkage.

Fengycins were purified mainly according to a flow of HCl precipitation, organic solution extract and reextract, gel permeation and silica gel chromatographies and RP-HPLC (Vanittanakom and Loeffler 1986). Our work developed a new preparation method of fengycin from culture broth.

Fengycins have an amphipathic structure leading to the formation of aggregates in water solution. Using this feature, we concentrated fengycins from culture broth by ultrafiltration. The long hydrophobic tails made fengycin homologs strongly interacted with the matrix of the hydrophobic interaction column. After this step, most contaminants in the filtrate were removed and the resulting fraction could be easily separated on reverse phase column to get purified fengycin homologs.

Loeffler et al. (1986) and the colleagues proved that fengycin was less toxic to the tested plants and protected them better from some filamentous pathogenic fungi than several peptide antibiotics such as iturin, suggesting fengycin and *B. subtilis* IB deserve further study on the capacity in controlling *F. graminearum*. Field tests using *B. subtilis* IB as the agent are now being performed in this laboratory.

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