Antifungal peptides produced by *Bacillus subtilis* for the biological control of aflatoxin contamination

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小野 誠*, 木村 宣夫*: *Bacillus subtilis* が生産するアフラトキシン産
生菌生育阻害物質の単離・同定

Summary

Iturin A, antifungal lipopeptide reported by Peypoux and Isogai, was isolated from submerged culture of *Bacillus subtilis* NK 330 and its growth inhibitory activity toward aflatoxigenic fungus *A. parasiticus* NRRL 2999 was investigated. At 50 ppm of iturin A in the medium, the toxin production was completely inhibited while the growth of *A. parasiticus* was partially restricted. In contrast, the same amount of cycloheximide showed no significant influence on the toxin production by this fungus.

Aflatoxins (AF) are secondary metabolites produced by *A. flavus* and *A. parasiticus*. They are potent hepatotoxins and carcinogens affecting wide variety of animal species¹⁾. Many substances, including pesticides^{2,3)}, spice⁴⁾ and extract of plants⁵⁾, were screened for the prevention of AF contamination in foods and feeds. Some microorganisms were also tested for the same purpose. Cuero⁶⁾ and Wicklow⁷⁾ have reported the influences of various microorganisms on the growth and the toxin production of aflatoxigenic fungi, but in all cases, practical inhibiting potency has not been feasible. In the course of search for the potent antagonist toward *Aspergilli*, a strain of *B. subtilis* named NK 330 found to be markedly inhibited the growth and the AF production of *A. flavus* and *A. parasiticus*⁸⁾. In the following, the isolation and the identification of this antifungal substance is described.

Materials and Methods

Microorganisms *Aspergillus flavus* strain NRRL 3357 and *A. parasiticus* strain NRRL 2999 were used for monitoring antifungal activity. *Bacillus subtilis* strain NK 330 was isolated from Japanese soil⁹⁾.

Reagent The specimen of iturin A¹⁰⁾ was kindly supplied by F. Peypoux of Claude Bernard University, France. Cycloheximide was purchased from Nacalai Tesque Co., Japan.

Media Potato-dextrose agar was used for the stock culture of aflatoxigenic fungi. For the assay of antifungal activity, serial plate dilutions of testing samples were made by potato-dextrose agar (Difco).

Spectral analysis FAB-MASS: JMS-DX 303 (JEOL). ¹H-NMR: Bruker AM 600. The

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chemical shift was determined by reflecting to an internal standard of tetramethylsilane.

Bioassay Serial plate dilution method was used for the assay of growth and AF production of *A. parasiticus* NRRL 2999. Test solution was sterilized by filtration and added to autoclaved PDA under 60°C. After the agar was set and surface was dried, one drop of spore suspension (2×10^6 spores/ml) of *A. parasiticus* was inoculated at the center of the plate (90 mm I.D.) and incubated at 25°C for 7 days. The diameter of colony was measured and AF was analyzed with HPLC according to the authentic method¹⁰.

Production and purification of the active substance *B. subtilis* NK 330 was preincubated in potato-dextrose medium (pH 6.8) for 24 hours in shake culture (100 r.p.m., 30°C). A 50 ml of this culture was used to inoculate 10 L-fermenter containing 7 L of potato-dextrose medium (pH 6.8). Submerged culture was performed at 30°C, under stirring (300 r.p.m.) and aeration (2 L/min) for 3 days. FS-F-20 (Dow Chem. Co.) was used as anti-form agent. The broth was centrifuged to eliminate cell bodies. The supernatant was concentrated to 300 ml with rotary evaporator. Then, 1.2 L of ethanol was added to the concentrated supernatant and stirred vigorously for several hours. The precipitated impurities were removed by centrifugation and thoroughly extracted twice with 1.5 L of 80% ethanol. The supernatant and the extracts were combined and evaporated to dryness. The residue was dissolved with 500 ml of deionized water. This solution was acidified to pH 3.0 with conc. HCl to precipitate active substance and left for several hours at 4°C. The resulting precipitate was collected and dissolved in adequate volume of dil. NH_4OH . This acidification step was repeated until yellowish pigment was removed. Finally obtained precipitate was dissolved in methanol and chromatographed on Sephadex LH-20 column (58 cm $\times$ 2.6 cm I.D.) with methanol as eluting solvent. The biologically active fraction was collected and evaporated. This fraction was applied to a silica gel column equilibrated with eluting solvent (silica gel 60, 70-230 mesh, Nacalai Tesque Co., column size 35 cm $\times$ 4.7 cm I.D.). Elution was carried out with ethanol:acetic acid:water (85 : 5 : 10, v/v/v). The collected active fraction was finally purified by using HPLC on a C18 column (μ -bondasphere C18 150 mm $\times$ 19 mm I.D., Waters Assoc.). Elution was carried out with a linear gradient of acetonitrile and water, going from 38 to 45% acetonitrile at 40 min. Total flow rate for the solvent system was 9.0 ml/min.

Results and Discussion

Six active peaks named F1 to F6 eluted at the retention times of 19.4, 25.9, 27.1, 28.9, 35.9 and 37.2 min (Fig. 1). About 77 mg of active substances were finally obtained from 7 L of culture broth. Isolated active substances were soluble in methanol and aqueous ethanol, but practically insoluble in other organic solvent. They were also soluble in alkaline water. Ninhydrin reaction was negative, but after hydrolyzed by HCl, turned positive. Since these properties were closely similar to those of iturin A^{9,11} isolated fractions were spotted on silica gel TLC plate (silica gel 60 F254, Merck) with specimen of iturin A and developed with various solvent systems. Table 1 shows that R_f values of all fractions were identical with those of iturin A. Amino acid analyses by TLC of the acid hydrolyzates of six frac-

Fig. 3. FAB-MS spectrum of F1. Molecular ion peak at 1043 ($M+H$) was observed.
(matrix: glycerine)

Fig. 4. ^1H -NMR spectrum of F1. F1 was dissolved in CD_3OD . *1: tetramethylsilane;
*2: signals derived from methanol (3.34, 4.88 ppm).

Table 2. The coupling pattern of the signals of terminal CH₃ of lipophilic β -amino acid in ¹H-NMR

	F1	F2	F3	F4
δ ppm (CH ₃)	0.9 (3H, t)	0.9 (6H, m)	0.9 (6H, d)	0.9 (3H, t)

Table 3. Inhibitory effect of iturin A and cycloheximide on aflatoxin production by *A. parasiticus*

Conc* ¹ (ppm)	Diameter of colony (mm)	AF* ² (ppm)
control	60.0	2.97
iturin A		
100	13.5	N. D.
50	46.0	N. D.
25	62.5	2.31
12.5	66.0	3.43
cycloheximide* ³		
50	50.0	1.1
25	55.0	1.54
12.5	58.0	2.26

*¹: concentration in PDA plate; *²: AF B₁, B₂, G₁, G₂; *³: cycloheximide, dissolved in dimethylformamide (20 mg/ml).

with iturin A-2 to A-5, respectively. From the elution profile and the amino acid constituent, F5 and F6 also seem to be iturin A. But mass spectra and ¹H-NMR spectra of F5 and F6 were failed to obtain because of poor yield of these fractions.

The influence of prepared iturin A on AF production was tested and compared with that of cycloheximide (Table 3). Iturin A showed completely inhibition of AF synthesis by *A. parasiticus* NRRL 2999 after 7 days incubation, though the growth of the fungus was partially inhibited. Cycloheximide was reported to enhance AF production by non-aflatoxigenic strain of *A. flavus*¹⁸⁾, but no significant influence on AF synthesis with *A. parasiticus* was detected in this study. Furthermore, iturin A was proved to have more potent antifungal activity toward *A. parasiticus* than cycloheximide. We failed to examine the influence of each isolated fraction on the AF biosynthesis because of poor yield of these fractions. Minimum inhibitory concentration (MIC) of each fraction toward *A. parasiticus* NRRL 2999 was examined up to 150 ppm with plate dilution method on PDA. Even if the concentration of these fractions in PDA plate was 150 ppm, these fractions could not completely inhibit the growth of *A. parasiticus* but the diameter of the colony was reduced to 15-20% of that of control. No significant difference was observed on this reduction of the growth rate among the fractions. Iturin A seemed to have fungistatic activity toward *A. parasiticus* rather than fungicidal activity and this fungistatic nature may result in the inhibition of the AF biosynthesis. Besson¹⁴⁾ reported that the site of the action of iturin A on yeast cell was the cytoplasmic membrane and an important modification of cell permeability was the principle of the action. Iturin A is considered to have low toxicity and low

allergenic properties in topical applications¹⁵⁾. Recently, iturin A was isolated as active principle in biological control of peach brown rot with *B. subtilis*¹²⁾. To apply iturin A for the control of AF contamination, further biological approach is currently on progress.

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