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Isolation and Identification of Antifungal Substances Produced by *Fusarium* sp. BYA-1

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Fusarium sp. BYA-1 균주가 생성하는 항진균성 항생물질의 분리 및 동정

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ABSTRACT : Three antifungal substances effective against various plant pathogens were isolated from the potato dextrose agar culture of an isolate of *Fusarium* sp. (BYA-1) isolated from barley. The three antibiotics were extracted and purified by using silica gel column chromatography and preparative high performance liquid chromatography (HPLC), and antibiotic efficacy was assayed on *Phytophthora capsici*. In order to identify the antibiotics, they were analyzed by instrumental analysis such as melting point determination, UV spectroscopy, mass spectrometry, and NMR spectrometry. The three antibiotics were identified as fusarielin A, enniatin B, and enniatin B_1. Of the antibiotics, fusarielin A showed the strongest antifungal activity against various fungi tested, and had minimal inhibitory concentration (MIC) values below 40 μg/ml. This is the first report that a *Fusarium* species coproduces structurally different antifungal antibiotics, fusarielin A and enniatins.

***Key words* : antifungal substance, *Fusarium*, fusarielin A, enniatin B, enniatin B_1.**

Fusarium species are spread over the world as saprophytes, soil inhabitants, and parasites of many plants. Many *Fusarium* species produce a number of secondary metabolites, which elicit physical and pharmacological responses in microbes, plants, and animals. Based on biological activity, the metabolites can be grouped into pigments, mycotoxins, antibiotics, and phytotoxins (7, 9, 11, 18, 21, 25).

As for the antibiotics produced by the genus *Fusarium*, many compounds including enniatins, equisetin, fusarielins, cyclosporins, avenacin, fusarubin, javanicin, lateropyrone, and beauvericin have so far been isolated (4, 8, 10, 14, 16, 27). Enniatins are hexadepsipeptides with alternating residues of 2-hydroxyisovaleric acid and branched *N*-methyl amino acids (26). A total of nine enniatin analogues have been proposed by either chemical or microbiological synthesis (2, 6, 7, 26, 28). They are produced by fermentation of various strains of fusaria, including *Fusarium acuminatum*, *F. avenaceum*, *F. compactum*, *F. lateritium*, *F. oxysporum*, *F. scirpi*, *F. sambucinum*, and *F. tricinctum* (2, 5, 6, 7, 20, 26, 28). The antibiotics show antimicrobial activities against *Mycobacterium phlei*, *Bacillus subtilis*, and *Staphylococcus aureus in vitro* at concentrations below 1 μg/ml, and also show insecticidal (13) and phytotoxic properties (2, 7).

Recently, a new antifungal antibiotic, fusarielin A, and three related compounds B, C, and D were isolated from a culture of *Fusarium* sp. and characterized (17). Fusarielin A showed a strong antifungal activity against *Aspergillus fumigatus*, *Alternaria kikuchiana*, *Colletotrichum lindemuthianum*, *F. nivale*, *Helminthosporium oryzae*, and *Pyricularia grisea*, and had minimal inhibitory concentration (MIC) values below

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50 μg/ml.

Antibiotics produced by microorganisms have been extensively studied owing to the possible usage of the metabolites directly as agrochemicals with highly selective activity against some plant pathogens and with no public hazard or as lead molecules for the synthesis of new chemical fungicides. During our screening of new antifungal antibiotics produced by *Fusarium* isolates, we obtained one *Fusarium* sp. isolate (BYA-1) showing a potent antifungal activity against *Phytophthora capsici*, which causes the phytophthora blight of red pepper. The isolate coproduced structurally different antifungal antibiotics, fusarielin A and enniatins. This paper describes the isolation, identification, and antifungal activity of the antibiotics.

MATERIALS AND METHODS

***Fusarium* isolate.** An isolate of *Fusarium* sp. (BYA-1) was obtained from a barley grain sample collected in Yangyang district, Kangwon province in 1990. The isolate showed antifungal activities against several plant pathogens such as *Phytophthora capsici*, *Alternaria alternata*, *Botrytis cinerea*, and *Pyricularia grisea* by the paper disc method (3). Stock culture of the isolate was single spore-isolated, maintained on moist autoclaved soil, and stored at −15°C.

Extraction and purification of antifungal substances. A total of 200 Petri dishes (diameter, 90 mm), containing about 20 ml of potato dextrose agar (PDA) medium for each, were autoclaved for 15 min at 121°C. The medium was inoculated with mycelium plugs from a 5-day-old PDA plate of the fungus. The PDA plates were incubated for 20 days at 25°C. The mycelial mass and media were harvested and divided into five subsamples. Each subsample was placed in a 3-liter flask and successively extracted three times with methanol (totalling 8 liters). The extracts were filtered through Whatman no. 2 filter paper and evaporated to dryness. The methanol extract was dissolved in 500 ml of acetonitrile and defatted twice with an equal volume of *n*-hexane. The acetonitrile phase was concentrated to dryness and then dissolved in 500 ml of distilled water. The aqueous phase was partitioned twice with an equal volume of chloroform, and the organic phase was concentrated to dryness. The chloroform extract was dissolved in 10 ml of chloroform and loaded onto a silica gel column (5×38 cm) containing 200 g of silica gel (Kiesel gel 60, 70/230 mesh; E. Merck, Darmstadt, Germany). The column was eluted with chloroform-methanol (99:1, 19:1, 9:1, 3:1, 1:1, v/v; each 300 ml). The eluate was collected in 10 ml fractions with a fraction collector. Each fraction was monitored by TLC and reduced to five fractions F1, F2, F3, F4, and F5, which were bioassayed with *P. capsici* by the paper disk method (3). The active fraction F3 (13 g) was dissolved in 10 ml of ethyl acetate-*n*-hexane (3:1, v/v) and loaded onto a silica gel column (3.6×60 cm) containing 200 g of silica gel (Kiesel gel 60, 230/400 mesh; E. Merck). The column was eluted with ethyl acetate-*n*-hexane (3:1, v/v) and the eluate was reduced to four fractions F31, F32, F33, and F34, which were bioassayed with *P. capsici*. The active fractions F32 (3.5 g) and F33 (2.3 g) were finally purified by preparative high performance liquid chromatography (HPLC; Waters Delta Prep 4000; Millipore Co., Milford, USA) on a Waters RCM mode column equipped with μBondapak C_{18} (25×100 mm). F33 was eluted with methanol-water (65:35, v/v) at a flow rate of 10 ml/min and all peaks were detected at 240 nm with Waters 486 tunable absorbance detector (Millipore Co.). The antifungal substance FST-01 (1.2 g) was purified as colorless solid. F32 was eluted with methanol-water (80:20, v/v) and all peaks were detected at 220 nm. Two antifungal substances FST-02 (1.5 g) and FST-03 (0.8 g) were purified as colorless solids.

Purity of the antifungal substances. The purified antibiotics were analyzed on TLC plates (UV, 254 nm) with ten different developing solvent systems (Table 1).

Table 1. TLC resolution of FST-01, FST-02, and FST-03 isolated from *Fusarium* sp. BYA-1 in various solvent systems[a]

Solvent system	Ratio by volume	R_f		
		FST-01	FST-02	FST-03
Chloroform-methanol	9:1	0.74	0.74	0.76
Chloroform-methanol	19:1	0.71	0.70	0.77
Chloroform-acetone	3:2	0.62	0.62	0.62
Ethyl acetate-*n*-hexane	3:1	0.87	0.90	0.90
Ethyl acetate-toluene	3:1	0.81	0.87	0.84
Benzene-acetone	3:1	0.52	0.54	0.54
Benzene-acetone	1:1	0.78	0.80	0.80
Toluene-methanol	7:3	0.62	0.62	0.62
Acetonitrile-water-benzene	45:3:2	0.96	0.98	0.98
Toluene-acetone-methanol	5:3:2	0.79	0.81	0.81

[a] The developed TLC plates were examined under UV light (254 nm) for the quenching spots.

The antibiotics were also analyzed by HPLC, and the following equipment and conditions were used; column, Zorbax C_{18} (4.6×150 mm); flow rate, 1 ml/min; mobile phase, methanol-water (65:35, v/v); UV detection at 240 nm.

Color reactions of the antifungal substances. FST-01, FST-02, and FST-03 were chromatographed on TLC plates (0.25 mm thick) and developed in chloroform-methanol (9:1, v/v). Seven reagents were used to determine their reactions with the antibiotics on TLC plates (Table 2). The color changes of antifungal antibiotics tested were observed after their treatments with the spraying reagents and heating at 110°C for 10 min (15).

Spectral measurements. The melting points of FST-01, FST-02, and FST-03 were determined with a Fisher-Johns melting point apparatus (Fisher Chemical Co., Chicago, USA) without correction. The UV spectra of the three antibiotics in a methanol solution were recorded on a Hitachi 340 double beam spectrophotometer. Low-resolution (LR) mass spectra were recorded on a double-focusing high-resolution (HR) mass spectrometer (JEOL JMS-AX505; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV. The samples were introduced via a direct-insertion probe. The LR electron impact (EI) mass spectra were recorded at 70 eV and a source temperature of 200°C. The LR-chemical ionization (CI) analysis was carried out with methane as the reagent gas, at a source pressure of about 61 Pa. The filament electron energy was 200 eV and the source temperature was 150°C. The direct-insertion probe was heated to 100°C in the EI and CI analysis. The accurate molecular mass of FST-01 was determined by using software peak matching at a resolution of 8,000.

^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL NMR spectrometer (400 MHz; JEOL Ltd.). The NMR spectra of FST-01 were measured at 50°C in dimethyl sulphoxide-d_6, since the NMR measurements of FST-01 at room temperature gave poor resolution, and referenced to solvent signals. The NMR spectra of FST-02 and FST-03 were measured at room temperature in chloroform-d_1 and referenced to tetramethylsilane (TMS).

Antibiotic activities of the antifungal substances. The minimum inhibitory concentrations (MICs) of the three antibiotics against several fungi were estimated by agar dilution assay. The fungi used are as follows; *Alternaria alternata*, *Aspergillus niger*, *Aspergillus oryzae*, *Phytophthora capsici*, *Helminthosporium* sp., and *Pyricularia grisea*. Each antibiotic substance was dissolved in acetone. Amounts up to 1 ml of the solution were added to 99 ml of PDA to provide the desired two-fold dilution test concentrations, 80 μg/ml, 40 μg/ml, 20 μg/ml, 10 μg/ml, and 5 μg/ml. The 5-day-old mycelial plugs were inoculated on the PDA plates containing different concentrations of each antibiotic substance. The MICs of the antibiotics against each test fungus were recorded as such for the minimal concentration that completely inhibited the growth of each test fungus after incubating at 25°C for 3~7 days.

Table 2. Color reactions of FST-01, FST-02, and FST-03 isolated from *Fusarium* sp. BYA-1[a]

Chemical reagent	Color reaction		
	FST-01	FST-02	FST-03
Aluminum chloride[b]	pink	–	–[c]
p-Anisaldehyde[d]	dark violet	–	–
2,4-Dinitrophenylhydrazine[e]	–	–	–
Hydrochloric acid[f]	pink	–	–
4-(*p*-Nitrobenzyl)pyridine/ tetraethylene-pentamine[g]	dark blue	–	–
Ninhydrin solution[h]	–	–	–
Sulfuric acid (20%) in methanol	orange	–	–

[a] Each antibiotic was chromatographed on TLC plates, developed in chloroform-methanol (9:1, v/v), dried, sprayed with each chemical reagent, and heated at 110°C for 10 min.

[b] Aluminium chloride (1%) solution in ethanol.

[c] No color reaction was observed.

[d] *p*-Anisaldehyde, 0.5 ml, was added to the mixture of 85 ml of methanol, 10 ml of glacial acetic acid, and 5 ml of concentrated sulfuric acid.

[e] A 10-ml volume of hydrochloric acid (36%) was added to a solution of 1 g of 2,4-dinitrophenylhydrazine in 100 ml of ethanol.

[f] Hydrochloric acid (36%) and ethanol were mixed in the volume ratio of 1:4.

[g] The plate was sprayed with a 3% (w/v) solution of 4-(*p*-nitrobenzyl)pyridine (NBP) in chloroform-carbon tetrachloride (2:3, v/v), dried, heated for 30 min at 110°C, cooled, and sprayed with 10% (v/v) tetraethylene-pentamine (TEPA) in chloroform-carbon tetrachloride (2:3).

[h] Ninhydrin (0.1%) in methanol.

RESULTS

Isolation of the antifungal substances. Three pure compounds FST-01, FST-02, and FST-03 were

Table 3. Physico-chemical properties of FST-01, FST-02, and FST-03 isolated from *Fusarium* sp. BYA-1

Property	FST-01	FST-02	FST-03
Appearance	colorless powder	colorless powder	colorless powder
Molecular weight	402	639	653
Molecular formula	$C_{25}H_{38}O_4$	$C_{33}H_{57}O_9N_3$	$C_{34}H_{59}O_9N_3$
Melting point	85~87°C	164~165°C	123~124°C
UV maxima (ε)	240 nm (22,800)	210 nm (23,600)	210 nm (23,600)

obtained from the culture of *Fusarium* sp. BYA-1. From 4 liters of the PDA culture, amounts of purified compounds were 1.2 g for FST-01, 1.5 g for FST-02, and 0.8 g for FST-03. The three antibiotics were subjected to TLC with ten different solvent systems. Each compound appeared as a single spot on TLC plates with various R_f values depending on the solvent system (Table 1). The purity of the three antibiotics was greater than 95% in the HPLC analysis.

Physico-chemical properties of the antifungal substances. FST-01 exhibited positive color reactions with chemical reagents such as *p*-anisaldehyde, hydrochloric acid, and 20% sulfuric acid (Table 2). The antibiotic also exhibited positive color reactions of pink with aluminum chloride, which reacts with hydroxy (−OH) or free amine ($-NH_2$) groups and dark blue with NBP/TEPA, which reacts with carboxylic acid or epoxy groups. But it did not exhibit any color reactions with ninhydrin solution reacting with amine groups, or 2,4-DNP reacting with active carbonyl groups. The results indicated the presence of hydroxy groups and epoxy or carboxylic acid groups in the structure of FST-01. FST-02 and FST-03 did not exhibit any color properties with the seven chemical reagents (Table 2).

The several physico-chemical properties of the three antibiotics are summarized in Table 3. Absorption maximum at 240 nm in the UV spectrum of FST-01 suggested the presence of two carbon-carbon double bonds conjugated in the structure of FST-01 (24). The UV spectra of FST-02 and FST-03 were almost identical, suggesting that the two compounds are structurally related each other.

All the three compounds were soluble in chloroform, ethyl acetate, acetone, benzene, methanol, and ethanol and insoluble in *n*-hexane and water.

Identification of the antifungal substances. The chemical structure of each antibiotic substance was determined by examining its mass and NMR spectra. The LR-EI mass spectrum of FST-01 displayed a strong

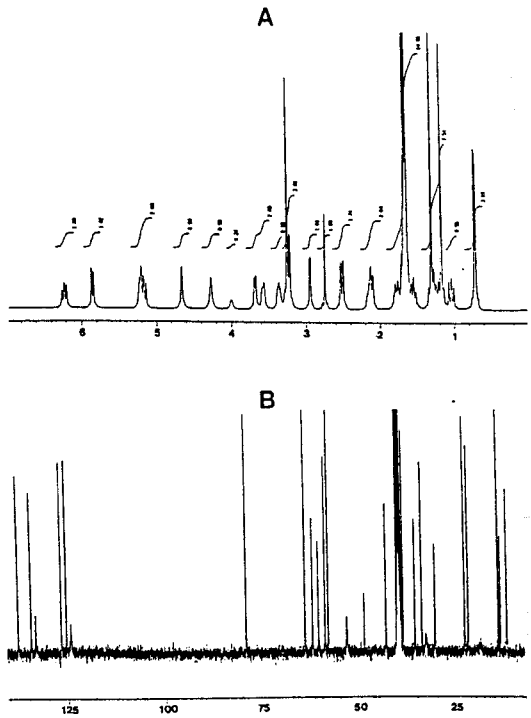

Fig. 1. ^1H- (A) and ^{13}C- (B) NMR spectra of FST-01. The spectra were measured at 50°C in dimethyl sulphoxide-d_6 and referenced to solvent signals.

molecular ion at *m/z* 402 and fragment ions at *m/z* 384, 354, 343, 325, 297, and 259. The cleavage from *m/z* 402 to 384 indicated the presence of a hydroxy group somewhere in the structure of FST-01. HR-EI mass spectrometry gave the molecular formula $C_{25}H_{38}O_4$ (M^+; cald, *m/z* 402.2771; found, *m/z* 402.2771). The molecular formula of FST-01 is identical to fusarielin A of antibiotics produced by *Fusarium* species. To unequivocally identify FST-01, ^1H- and ^{13}C-NMR spectra were obtained. The ^1H-NMR (Fig. 1A) indicates the presence of four secondary and two tertiary methyls. Four proton signals at 5.0 ppm to 6.5 ppm arise from the methines which participate in the carbon-carbon

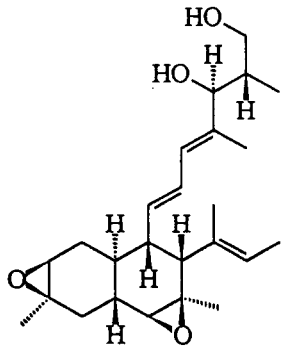

Fig. 2. Chemical structure of fusarielin A.

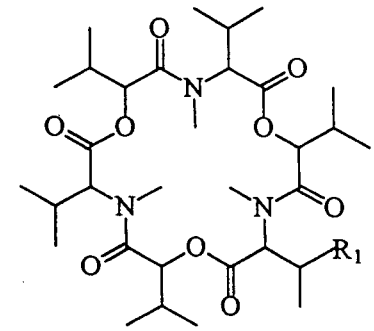

Fig. 4. Chemical structures of enniatin B (R_1=CH_3) and B_1 (R_1=CH_2CH_3).

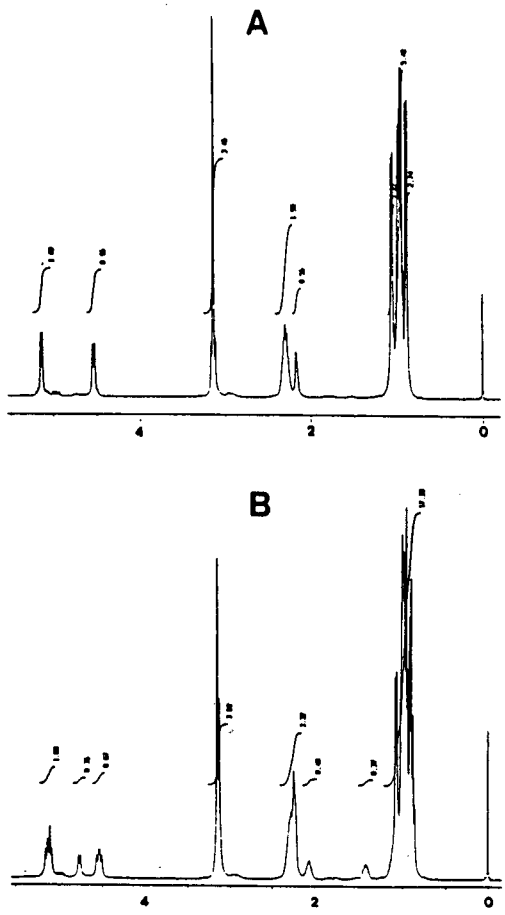

Fig. 3. ^{1}H-NMR spectra of FST-02 (A) and FST-03 (B). The spectra were measured at room temperature in chloroform-d_1 and referenced to TMS.

double bonds. The ^{13}C-NMR spectrum (Fig. 1B) of FST-01 showed 25 carbon signals. Six carbon signals at 125 ppm to 140 ppm and the 6 carbon signals at 55 ppm to 80 ppm are from the 6 carbons participating in the carbon-carbon double bonds and the 6 oxygenated carbons, respectively. The ^{1}H- and ^{13}C-NMR spectral data were completely identical to fusarielin A (17). Thus, FST-01 was identified as an antifungal antibiotic fusarielin A (Fig. 2).

The LR-EI mass spectrum of FST-02 showed a molecular ion peak at *m/z* 639 and fragment ions at *m/z* 556, 538, 496, 409, 296, 282, 196, and 86. The LR-EI mass spectrum was consistent with that of enniatin B (7), which is one of the antibiotics produced by *Fusarium* species. The strong [M+1]$^+$ ion peak at *m/z* 640 in LR-CI mass spectrum of FST-02 supported the molecular weight of FST-02. Both ^{1}H- (Fig. 3A) and ^{13}C-NMR spectra of FST-02 were consistent with enniatin B (28). Because there are the three repeating units in the molecule, only a third as many proton and carbon signals appeared as the molecular weight suggested. A doublet at 5.13 ppm was observed for the methine proton between the ester oxygen and the amide carbonyl. The methine proton between the amide nitrogen and the ester carbonyl was seen as a doublet at 4.53 ppm. The *N*-methyl protons are seen as a singlet at 3.14 ppm. Two signals at 2.26 and 2.17 ppm are from the methines on the isopropyl groups, and four doublet signals around 0.98 ppm arise from the methyl groups on the isopropyl groups. Thus, FST-02 was identified as enniatin B (Fig. 4).

The LR-CI mass spectrum of FST-03 had abundant signal only at m/z 654 as [M+1]$^+$. The LR-EI mass spectrum had abundant molecular ion at m/z 653 and fragment ions across the mass range. The fragmentation pattern of FST-03 was similar to enniatin B and the molecular weight was consistent with those of enniatin B_1 and B_4 (28). In order to verify the identity

Table 4. Minimum inhibitory concentrations of fusarielin A, enniatin B, and enniatin B_1 against various microorganisms[a]

Test organism	MIC (μg/ml)		
	Fusarielin A	Enniatin B	Enniatin B_1
Alternaria alternata	40	40	40
Aspergillus niger	40	> 80	> 80
Aspergillus oryzae	5	40	40
Helminthosporium sp.	10	80	80
Phytophthora capsici	40	80	80
Pyricularia grisea	10	20	20

[a] The conventional agar dilution method for MIC was used.

of FST-03, ^{1}H- and ^{13}C-NMR spectra were obtained. Both ^{1}H- (Fig. 3B) and ^{13}C-NMR spectra of FST-03 were identical to those of enniatin B_1. Because an isopropyl group in one repeating unit is replaced with an isobutyl group, the signals were somewhat broadened and some more proton and carbon signals appeared. A doublet at 4.77 ppm was observed for the proton of a methine, to which an isobutyl group instead of isopropyl group is attached and which is between the ester oxygen and the amide carbonyl. A multiplet at 1. 42 ppm is from the methylene on an isobutyl group. Thus, FST-03 was identified as enniatin B_1 (Fig. 4).

Antibiotic activities of the antifungal substances. The MICs of the three antibiotics against six fungi are summarized in Table 3. Of the three antibiotics, fusarielin A showed the strongest antifungal activity to the fungi tested, with MIC values below 50 μg/ml. The MICs of fusarielin A were 5 μg/ml for *Aspergillus oryzae*, 10 μg/ml for *Helminthosporium* sp. and *Pyricularia grisea*, and 40 μg/ml for *Alternaria alternata*, *Aspergillus niger*, and *Phytophthora capsici*. The anfungal activities of enniatin B and B_1 were similar each other. *A. niger* was not inhibited by 80 μg/ml of enniatin B and B_1 and the MICs of two antibiotics for the other fungi were 20 μg/ml to 80 μg/ml.

DISCUSSION

Fusarium sp. BYA-1 isolate produced three antifungal antibiotics. Those antifungal antibiotics were purified by solvent extraction and various chromatographies.

The purified antibiotics were identified as fusarielin A, enniatin B, and enniatin B_1 mainly by mass and NMR spectral data. Fusarielin A was recently isolated from the culture of a *Fusarium* sp. K432 with three related compounds, fusarielin B, C, and D (17). Kobayashi *et al.* (17) reported that fusarielin A showed an antifungal activity by interfering microtubule function of fungal mycelia. The compound also had a weak cytotoxicity against HeLa S3 and NCI-H69 cells.

Enniatins are the representative compounds of the antibiotics produced by various species of *Fusarium* and nine derivatives, namely enniatin A, A_1, A_2, B, B_1, B_2, B_3, B_4, and C have so far been isolated (2, 6, 7, 26, 28). In addition to antibiotic activity, the enniatins also have insecticidal and phytotoxic activities. The biological activities appears to be related to their ionophoric properties (22, 23). Enniatins are known to uncouple oxidative phosphorylation in isolated mitochondria, a reaction mediated by induction of an energy-dependent accumulation of potassium ions (1). They also affect water uptake by cells in tomato shoots, causing the symptoms of toxic wilt (12). Usually, an isolate of *Fusarium* species produces a few major enniatins with some minor derivatives. However, *Fusarium* sp. BYA-1 isolate did not produce any of other enniatins except enniatin B and B_1.

Fusarium species produce various antibiotics, such as enniatins, equisetin, beauvericin, cyclosporins, fusarielins, etc. In general, an isolate of *Fusarium* species produces an antibiotic as a major with its minor derivatives (6, 7, 8, 14, 17). But *Fusarium* sp. BYA-1 isolate used in this study coproduced in large amounts two structurally different kinds of the antibiotics fusarielin A, enniatin B, and enniatin B1. This is the first report that *Fusarium* species coproduces fusarielin A and enniatins.

Antagonistic microorganisms are used for the control of plant diseases by the use of the microorganism directly as a biological control agent, by the use of metabolites produced by fermentation as biochemical fungicides, or by the use of microbiologically produced compounds as lead molecules for the synthesis of new chemical fungicides (19). The use of fusarielin A and enniatins directly as agrochemicals is almost impossible because of their toxicities to animal cells, plants, or insects (12, 17, 26). However, *Fusarium* sp. BYA-1 isolate itself can be applied as a biological agent against phytopathogens causing soil-borne diseases such as *Phytophthora capsici*. Further studies *in vivo* and in the field are needed for the practical use of the antagonistic fungus in the control of the plant diseases.

요 약

보리로부터 분리한 *Fusarium* sp. BYA-1균주의 감자한천배지 배양체로부터 여러 식물병원곰팡이에 길항력을 나타내는 세 개의 항생물질을 분리하였다. 추출한 세 개의 항생물질은 silica gel관 크로마토그래피와 분취 HPLC, 그리고 *Phytophthora capsici* 검정을 이용하여 정제하였다. 이들 분리한 항생물질들을 동정하기 위하여 융점 결정, 자외선흡광법, 질량분석 및 핵자기공명법 등의 기기분석을 실시하였다. 그 결과, 세 개의 항진균성 항생물질들은 fusarielin A, enniatin B, 그리고 enniatin B_1으로 각각 동정되었다. 분리한 세 개의 물질 중 fusarielin A가 공시된 곰팡이에 가장 강한 항균활성을 나타내었으며, 최소저해농도는 40 μg/ml이하였다. *Fusarium*속 균주가 구조적으로 다른 두 종류의 항진균성 항생물질인 fusarielin A와 enniatins을 동시에 생성한다는 것은 본 논문에서 처음으로 보고하는 것이다.

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