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FULL PAPER

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Production of enokipodins A, B, C, and D: a new group of antimicrobial metabolites from mycelial culture of *Flammulina velutipes*

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Abstract Antimicrobial compounds enokipodins A, B, C, and D were originally isolated from the culture filtrates of *Flammulina velutipes* mycelial culture. Analysis of antibacterial activity by the paper disk method and quantification of enokipodins A–D by high performance liquid chromatography (HPLC) showed that *F. velutipes* mycelia produced enokipodins A–D in their late growing phase. Great genetic variability in production of these compounds was observed among ten strains of *F. velutipes* in analyses of antimicrobial activity by the hole-plate diffusion method and quantification by HPLC. Enokipodins A–D demonstrated antimicrobial activity mainly against the gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*. Evaluation of minimum inhibitory doses (MIDs) showed that MIDs of enokipodins A and C for *B. subtilis* were as low as that of the penicillin G antibiotic.

Key words Antimicrobial activity · Enokipodins A–D · Enokitake · *Flammulina velutipes* · Sesquiterpenoid

Introduction

Compounds with medicinal properties have been isolated from *Flammulina velutipes* (Curt.: Fr.) Sing. Flammulin, an antitumor protein (Komatsu et al. 1963; Zhang et al. 1999), flammutoxin, a cytolytic protein (Lin et al. 1974; Tomita et al. 1998), FVA-L, a lectin that possesses hemagglutination activity (Tsuda 1979; Wang et al. 1998), and FIP-*fve*, an immunomodulatory protein (Ko et al. 1995) were isolated from the *F. velutipes* fruiting body. Yoshioka et al. (1973) obtained some antitumor polysaccharide fractions, i.e., EA_3, EA_5, and EA_6, from aqueous extract of the *F. velutipes* fruiting bodies. These polysaccharides showed high tumor inhibition against sarcoma 180 in vivo (Ikekawa et al. 1982; Ohkuma et al. 1983; Otagiri et al. 1983). Another polysaccharide, SFA1, also exhibited potent antitumor activity against sarcoma 180 in vivo but not in vitro (Leung et al. 1997). Proflamin, an antitumor glycoprotein, was isolated from *F. velutipes* mycelium culture by Ikekawa et al. (1985). We have isolated the sesquiterpenoids, enokipodins A–D (Fig. 1), from the 30–45 days culture filtrates as the major antimicrobial metabolites and elucidated their structures (Ishikawa 2001; Ishikawa et al. 2000, 2001). Here we report the production time-course, minimum inhibitory doses (MIDs), and antimicrobial spectra of enokipodins A–D and the variability in production of those compounds among ten isolates of *F. velutipes* tested.

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Materials and methods

Microorganism species and isolates

The ten *F. velutipes* isolates, Fv-4, -5, -14, -25, -33, -34, -35, -36, -37, and -38, used in this study are kept in the culture collection of the Laboratory of Forest Resource Biology, Graduate School of Agriculture, Hokkaido University. Isolates Fv-4, -5, -14, -25, and -35 are wild type, the origins of Fv-33 and -34 are uncertain, and Fv-36, -37, and -38 originated from commercial sources (Table 1). The microorganisms used for testing the antimicrobial spectra are listed in Table 2. Fungal cultures were preincubated for 7–10 days at 25°C in the dark on malt extract agar (MEA) medium [3% malt extract (Difco, Sparks, MD, USA), 0.3% peptone (Merck, Frankfurt, Germany), and 1.5% agar (Difco), in distilled water], and then maintained at 4°C. Bacterial cul-

Fig. 1. Chemical structures of antimicrobial metabolites enokipodins A, B, C, and D and a hypothetical biosynthetic relation

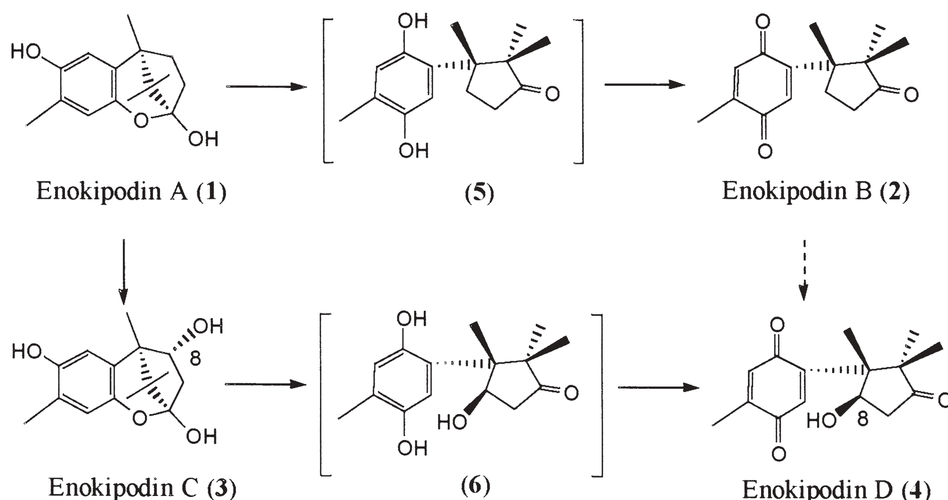

Table 1. Strain and sources of *Flammulina velutipes* used for testing the presence of enokipodins A–D

Strain	Type	Sources
Fv-4[a]	Wild	Hokkaido Forest products Research Institute, Asahikawa, Hokkaido, Japan
Fv-5	Wild	Campus of Iwate University, Iwate-ken, Japan
Fv-14	Wild	Syumarinai, Bifuka, Hokkaido, Japan
Fv-25	Wild	Kanayama (lakeside), Minami Furano, Hokkaido, Japan
Fv-33[b]		Kyushu Research Center Forestry and Forest Products Research Institute, Kurokami, Kumamoto-ken, Japan
Fv-34[b]		Kyushu Research Center Forestry and Forest Products Research Institute, Kurokami, Kumamoto-ken, Japan
Fv-35	Wild	Nopporo Forest Park, Nopporo, Hokkaido, Japan
Fv-36	Commercial	Kagawa Shiitake Co., Sakada, Miyagi-ken, Japan
Fv-37	Commercial	Akiyama Tanekin Kenkyusho, Koufu, Yamanashi-ken, Japan
Fv-38	Commercial	Kinokkusu Co., Sendai, Miyagi-ken, Japan

[a] All strains are kept in the culture collection of Lab. Forest Resource at the Graduate School of Agriculture, Hokkaido University
[b] Uncertain origin

tures were preincubated for 2–3 days at 37°C in the dark on brain heart infusion agar (BHIA) medium [3.7% brain heart infusion (Oxoid, Hampshire, England) and 1.5% agar (Difco), in distilled water], and then maintained at 4°C.

Culture conditions

Five disks (7mm in diameter) of the mycelia of each *F. velutipes* freshly grown on MEA were inoculated in Erlenmeyer flasks (300ml) containing 100ml malt extract broth (MEB) [3% malt extract (Difco) and 0.3% peptone (Merck) in distilled water, pH 4.5]. They were cultured at 25°C in the dark, under stationary conditions. For examination of antibacterial activity and mycelial dry weight, *F. velutipes* (Fv-4) mycelia were cultivated for 10, 20, 25, 30, 40, and 50 days in triplicate. Mycelia were cultivated for 55 days in triplicate to examine the production of enokipodins A–D in the ten *F. velutipes* isolates (see Table 1).

Metabolite extraction

The production time-course of enokipodins A–D in the culture filtrates of *F. velutipes* (Fv-4) and their productivity among ten *F. velutipes* isolates were examined as follows: a part of each culture filtrate (50ml) was extracted with 25ml EtOAc three times. The combined extracts were washed twice with 40ml saturated NaCl solution, dried over $MgSO_4$, and evaporated to give an oily residue, which was weighed and diluted with EtOAc. The mycelia in each flask was dried by a drying oven at 110°C until constant weight and weighed.

Antibacterial test by paper disk method

The paper disk method was used to evaluate the antibacterial activity of *F. velutipes* metabolites produced in mycelial cultures grown for different times. The liquefied BHIA medium (15ml, at about 40°C) was homogeneously seeded with about 10^5 colony-forming units/ml (CFU/ml) of the test bacterium in a Petri dish (9cm diameter). After

Table 2. Species and strains of microorganisms used for testing the antimicrobial spectra of enokipodins A–D

Species	Strain
Gram-positive bacteria	
Bacillus subtilis	LMA 0011
B. subtilis	IFO 12734
Staphylococcus aureus	AHU 1142
Gram-negative bacteria	
Escherichia coli	AHU 1714
E. coli	IFO 3301
E. coli	IFO 12113
Proteus vulgaris	AHU 1144
Pseudomonas fluorescens	AHU 1719
Yeasts	
Candida albicans	JCM 1542
Saccharomyces cerevisiae	AHU 3027
Filamentous fungi	
Aspergillus oryzae	AHU H-14
Cladosporium herbarum	AHU 9262
Hipocrea nigricans	AHU 31290
Mucor javanicus	AHU 3052
Penicillium roqueforti	AHU 8033
Spicellum roseum	JCM 10407
Sporothix schenckii	IFO 8158
Trichoderma harzianum	IFO 31292
T. polysporum	IFO 39322

LMA, Laboratory of Food Microbiology, Federal University of Viçosa, Brazil; IFO, Institute for Fermentation, Osaka, Japan (collection transferred to NBRC); AHU, Faculty of Agriculture, Hokkaido University, Japan; JCM, The Institute of Physical and Chemical Research (RIKEN), Japan

solidification of the BHIA medium, each paper disk (8 mm diameter) soaked with EtOAc extracts equivalent to 250 μl culture filtrates and dried under sterile condition was placed on the medium. These Petri dishes were kept at 4°C overnight, so that the metabolites could diffuse into the medium before bacterial growth. The plates were then incubated at 37°C for 18 h. The antibacterial activity was determined by measuring the diameter of the clear inhibition zone around each disk. To evaluate the minimum inhibitory doses (MIDs), 0, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 μg of enokipodins A and C, dissolved respectively in acetone (20 μl), was applied on each paper disk. The antibacterial activities of enokipodins A and C against *Bacillus subtilis* LMA 0011 were compared with two antibiotics, novobiocin (Sigma) and penicillin G (Wako, Osaka, Japan), and one synthetic compound, pentachlorophenol (PCP) (Wako). To evaluate the antimicrobial spectra, 25 and 50 μg enokipodins A–D were applied on paper disks. The experiments were performed in triplicate.

Antimicrobial test by the hole-plate diffusion method

The variability in production of enokipodins A–D among ten isolates of *F. velutipes* was tested by the hole-plate diffusion method. The liquefied BHIA medium (20 ml, at about 40°C) was homogeneously seeded with about 10^5 CFU/ml of the test bacterium in a Petri dish (9 cm diameter). After the mixture had solidified, 7-mm-diameter holes were punched with a cork borer and 0.1 ml each of the respective culture filtrate was poured into the hole. These Petri dishes were kept at 4°C overnight and then incubated at 37°C for 12–18 h. The antibacterial activity was determined by measuring the distance between a hole border and a clear inhibition zone border. This method is convenient to assess the relative antimicrobial activity of whole fungal metabolites produced in each culture medium without any extraction or fractionation procedure.

Metabolite quantification by high performance liquid chromatography (HPLC)

Production of the antimicrobial metabolites, enokipodins A–D, was analyzed by HPLC (pump, L-6320 Intelligent Pump; autosampler, AS-2000; detectors, L-4250 UV-VIS for 254 nm and L-4200 UV-VIS for 300 nm; Integrator, D-2500; Hitachi, Tokyo, Japan). Analytical conditions were as follows: column, YMC-Pack Polymer C18 (250 × 4.6 mm I.D.; YMC, Kyoto, Japan); eluent, 60% acetonitrile (CH_3CN); flow rate, 0.5 ml/min; detection, UV 300 nm for enokipodins A and C, UV 254 nm for enokipodins B and D.

HPLC sample preparation

The EtOAc extracts of the culture filtrate were concentrated to dryness. Each residue was dissolved in 1 ml 50% CH_3CN and applied to a C18 Sep-pak Plus cartridge (Sep-Pak Plus, short body type; Waters, Milford, MA, USA) which was eluted with 4 ml 50% CH_3CN. This eluate was evaporated to dryness, and the residue was dissolved in 2 ml CH_3CN, containing 1 mg of the internal standard, 2-methoxynaphthalene (Tokyo Kasei Kogyo, Tokyo, Japan). Usually, 5 μl of this sample was analyzed by HPLC.

Separation of authentic enokipodins A–D

Some combinations of chromatographic solvents, flow rates and, column were devised for separation of authentic enokipodins A–D. Chromatographic separation was performed favorably within 30 min using YMC-Pack Polymer C18 reversed-phase column (YMC), with a solvent system of 60% CH_3CN, at flow rate 0.5 ml/min.

Calibration curves

To obtain calibration curves, including the loss of enokipodins during the extraction process, enokipodins A and C (65, 125, 250, and 500 μg/ml acetone) as well as enokipodins B and D (32, 65, 125, and 250 μg/ml acetone) were added in 20 ml malt extract broth. Subsequently, each sample was extracted in the same manner as for culture filtrate extraction. The oily residue was dissolved in CH_3CN containing the internal standard to adjust in volume (2 ml) and analyzed following the conditions for HPLC described above.

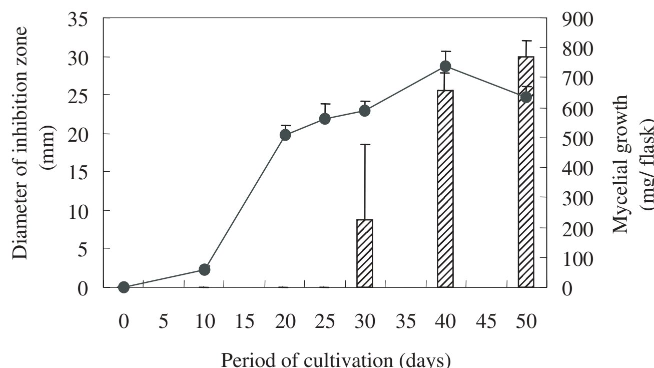

Fig. 2. Mycelial growth (*lines*) and antibacterial activity (*columns*) of *Flammulina velutipes*, isolate Fv-4. Antibacterial activity of culture filtrate against *Bacillus subtilis* (LMA 0011) was measured as diameter of the inhibitory zone around the paper disk soaked with EtOAc extract equal to 0.25 ml culture filtrate, by paper disk method

Results and discussion

Time-course of mycelial growth and antibacterial activity

Mycelial growth and antibactetial activities for 50 days of cultivation of *F. velutipes* (Fv-4) are shown in Fig. 2. The *F. velutipes* (Fv-4) growth was pronounced between 10 and 20 days, and maximal growth was reached on day 40. Antibacterial activity was detected only after 25 days of mycelial cultivation, and a progressive increase was observed until 50 days. These results show that *F. velutipes* mycelia produced antimicrobial compounds in their late growing phase.

Time-course of enokipodins A–D production

HPLC was applied to analysis of the time-course in enokipodins A–D production for *F. velutipes* (Fv-4) mycelial culture. The retention times (^{t}R) for enokipodins A, B, C, and D were 11.3, 13.8, 8.0, and 9.5 min, respectively. The ^{t}R for the internal standard, 2-methoxynaphthalene, was 26.9 min.

Calibration curves for enokipodins A–D were obtained from calculations of peak area ratios, that is, their HPLC peak areas in the aforementioned concentration divided by internal standard peak area. Thus, the calibration equations for enokipodins A–D were obtained as shown in Fig. 3A–D.

The production of the antimicrobial compounds began after 25 days of *F. velutipes* mycelial culture in the MEB medium (Fig. 4). The sum of the four compounds increased progressively to 55 days (1.975 mg/ml filtrate), in agreement with the result obtained in the antimicrobial assessment (see Fig. 2). However, this increase pattern differed between enokipodins. On day 30, the quantities of enokipodins A and C were practically equal (about 0.086 and 0.088 mg/ml filtrate, respectively). Nevertheless, production of enokipodin C increased sharply between 30 and 50 days and reached a plateau on day 55. A minor but similar increase pattern for enokipodin D was observed between 40 and 55 days, although quantities of the enokipodins A and B were not greatly altered (Fig. 4). This result suggests that the quantity of enokipodin A remains low (about 0.086 mg/ml culture filtrate) because this metabolite seemed to be a common precursor of the other metabolites, enokipodins B, C, and D (see Fig. 1). Enokipodin A was mainly oxidized by *F. velutipes* at the C-8 position to yield enokipodin C (1.037 mg/ml culture filtrate on day 55). We reported previously that enokipodins A and C were autoxidized to enokipodins B and D on silica gel thin-layer plates, via the keto-type tautomers illustrated as **5** and **6**, respectively, which has not yet been detected (see Fig. 1) (Ishikawa et al. 2000, 2001). A small part of enokipodin A was oxidized to yield enokipodin B (0.0026 mg/ml culture filtrate on day 55). Similarly, enokipodin C was oxidized to enokipodin D (0.834 mg/ml culture filtrate on day 55). The possibility that enokipodin B is oxidized by the fungus to yield enokipodin D is not ruled out (Fig. 1).

Production of enokipodins A–D by several *F. velutipes* strains

The antimicrobial activity of the culture filtrates of ten *F. velutipes* strains was investigated by the hole-plate diffusion method against *B. subtilis* LMA 0011. The strains were cultivated in MEB for 40, 55, and 70 days. Among the ten strains tested, the strains Fv-4, Fv-14, and Fv-35 showed notable antibacterial activity even in 40-day culture (Fig. 5). The strains Fv-25, Fv-33, Fv-34, and Fv-38 exhibited antibacterial activity only in 55- or 70-day cultures (Fig. 5). On the other hand, no inhibition zone was observed in the assays using the mycelial cultures of strains Fv-5, Fv-36, and Fv-37, even after 70 days (Fig. 5). Enokipodins A–D in the culture filtrates on day 55 were quantified by HPLC. Considerable amounts of enokipodins A and C were detected in the extracts of strains Fv-4, Fv-14, Fv-35, and Fv-38 culture filtrate, and a small quantity of enokipodin C was also observed in the culture of strains Fv-25 and Fv-34 (Fig. 6). These results indicate that the *F. velutipes* strains tested did not always produce enokipodins A–D. The different productivity of enokipodins between strains is clearly related with those antimicrobial activities (see Figs. 5, 6). Observation of this genetic variability will contribute to the selection of isolates with greater enokipodin A–D production and genetic breeding of strains more resistant to contamination in commercial *F. velutipes* production.

Determination of the minimum inhibitory doses of enokipodins A and C

The MIDs of enokipodins A and C against *B. subtilis* LMA 0011 were 3.12 and 6.25 μg, respectively. Under the present conditions, enokipodins A and C showed lower and the same MID, respectively, in comparison with penicillin G (Table 3). The MIDs for novobiocin and PCP against the strain were 0.78 and 1.56 μg, respectively. It should be taken into consideration that in antibiotic resistance monitoring

Fig. 3A–D. Calibration curves for enokipodins A–D

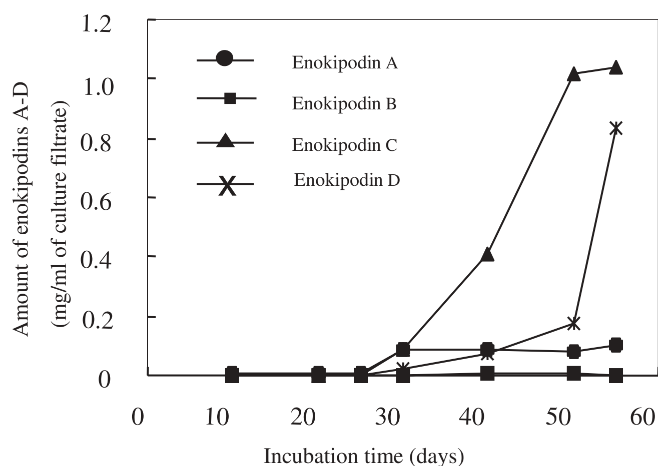

Fig. 4. Time-course production of enokipodins A, B, C, and D in mycelial culture of *F. velutipes*, isolate Fv-4 (average of three replications)

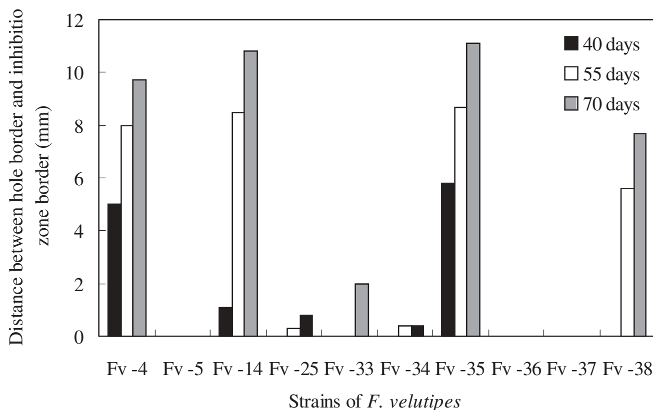

Fig. 5. Antibacterial activities of mycelial culture filtrates of ten *F. velutipes* strains. Each strain was cultivated in malt peptone broth for 40, 55, and 70 days, and antibacterial activity of culture filtrate (100 μl) against *B. subtilis* LMA 0011 was examined by hole-plate diffusion method (average of three replications)

of bacteria, the quantity of antibiotic loaded onto the paper disk varies drastically (2–300 μg/disk), but usually 10–30 μg per paper disk is used (Bauer et al. 1966; Tavares 2001). This study shows that enokipodins A and C have potential antimicrobial activity.

Antimicrobial spectra of enokipodins A–D

The gram-positive bacteria *Bacillus polymixa* and *Bacillus licheniformis* were identified to be the cause of a symptom called "stop-shoujyou" and "negusare-shoujyou" in the cultivation of *F. velutipes* (Kitamoto et al. 1987). Table 4 shows the antimicrobial spectra of the enokipodins A–D determined by the paper disk method. The enokipodins A–D were highly active against the gram-positive bacteria *B. subtilis* and *Staphylococcus aureus*, whereas gram-negative bacteria, yeasts, and filamentous fungi were not affected by the amount of 50 μg/paper disk. Gram-negative bacteria are often intrinsically resistant to the action of antibiotics because of the presence of an outer membrane that acts as a barrier to antimicrobial compound penetration into a bacterial cell (Tortora et al. 2000; Tavares 2001). Enokipodins A–D were narrow range spectra antimicrobial compounds. The negative results obtained by using the fungus *Cladosporium herbarum* on the paper disk method were in contrast to those in the thin-layer chromatography (TLC) bioautography method where clear inhibition spots were observed (Ishikawa et al. 2000, 2001). The sensitivity of the test fungus may be quite different when it grows on TLC plates or on wet agar plates.

Thus, there is great variability among *F. velutipes* isolates for enokipodins A–D antimicrobial production. The wild strains Fv-4, Fv-14, and Fv-35 presented greater productivity. Studying isolate Fv-4, it was ascertained that the enokipodins A–D compounds began to be produced in the late stage of mycelial growth and thus antimicrobial activity was not observed at the beginning of *F. velutipes* mycelial growth in MEB. Enokipodins A–D showed activity mainly against the gram-positive bacteria *B. subtilis* and *S. aureus*. The MID showed that *B. subtilis* was sensitive to enokipodins A and C even at dosages as low as that of the well-known antibiotic penicillin G.

In a preliminary test, enokipodins A and C were detected by gas chromatography–electron impact mass spectroscopy (GC-EI-MS) in the extract prepared from the fruiting bodies of *F. velutipes* Fv-4 fruited in continuous illumination (data not shown). The present results indicate that some *F. velutipes* strains produce notable quantities of antimicrobial metabolites in the stationary phase of mycelial growth. It is presumable that *F. velutipes* is in constant competition for substrates with other microorganisms and subject to attacks by disease-causing microorganisms, so enokipodins A–D may play an important role in the survival and production of *F. velutipes* fruit bodies in the wild and in commercial cultivation.

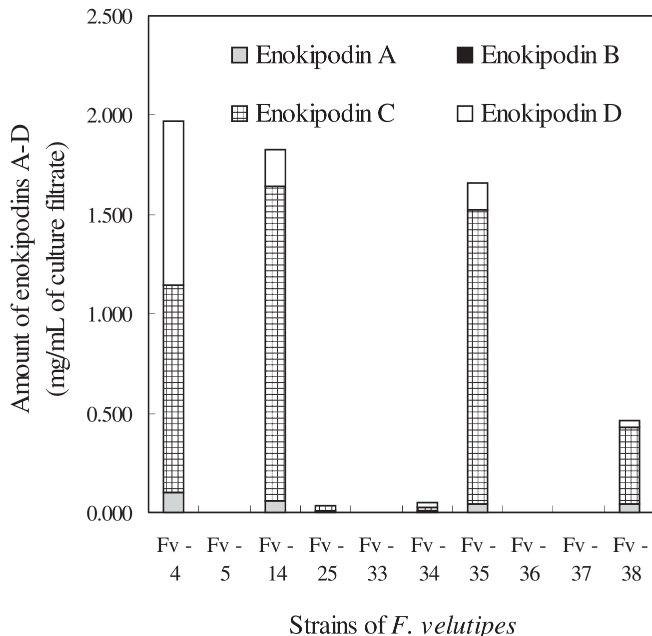

Fig. 6. Production of enokipodins A–D by mycelial culture of ten strains of *F. velutipes* in 55 days of cultivation (average of three replications)

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Table 3. Minimum inhibitory dose (MID) against *Bacillus subtilis* LMA 0011 of enokipodins A and C in comparison with those of novobiocin, penicillin G, and pentachlorophenol (PCP)

	0 μg	0.39 μg[a]*	0.78 μg	1.56 μg	3.12 μg	6.25 μg	12.5 μg	25 μg
Enokipodin A	–	–	–	–	12.2[b]	16.8	21.7	25.0
Enokipodin C	–	–	–	–	–	12.8	17.5	20.0
Novobiocin	–	–	10.2	14.8	19.0	22.7	25.8	28.0
Penicillin G	–	–	–	–	–	24.5	26.8	33.3
PCP	–	–	–	10.8	15.7	21.3	26.3	30.5

The experiments were done in triplicate

−, No inhibition

[a] Dose amount loaded onto 8-mm-I.D. paper disk

[b] Diameter of inhibitory zone (mm)

Table 4. Spectrum of antimicrobial activity of enokipodins A–D

Species	Enokipodin A		Enokipodin B		Enokipodin C		Enokipodin D	
	25 μg[a]	50 μg	25 μg	50 μg	25 μg	50 μg	25 μg	50 μg
Gram-positive bacteria								
Bacillus subtilis LMA 0011	+++	+++	+	++	+	++	±	+
B. subtilis IFO 12734	++	+++	nt	nt	+	++	nt	nt
Staphylococcus aureus AHU 1142	+	++	−	±	+	++	−	±
Gram-negative bacteria								
Escherichia coli AHU 1714	−	−	−	−	−	−	−	−
E. coli IFO 3301	−	−	nt	nt	−	−	nt	nt
E. coli IFO 12113	−	−	nt	nt	−	−	nt	nt
Proteus vulgaris AHU 1144	−	−	nt	nt	−	−	nt	nt
Pseudomonas fluorescens AHU 1719	−	−	nt	nt	−	−	nt	nt
Yeasts								
Candida albicans JCM 1542	−	−	−	−	−	−	−	−
Saccharomyces cerevisiae AHU 3027	−	−	−	−	−	−	−	−
Filamentous fungi								
Aspergillus oryzae AHU H-14	−	−	nt	nt	−	−	nt	nt
Cladosporium herbarum AHU 9262	−	−	−	−	−	−	−	−
Hipocrea nigricans AHU 31290	−	−	nt	nt	−	−	nt	nt
Mucor javanicus AHU 3052	−	−	nt	nt	−	−	nt	nt
Penicillium roqueforti AHU 8033	−	−	nt	nt	−	−	nt	nt
Spicellum roseum JCM 10407	−	−	nt	nt	−	−	nt	nt
Sporothix schenckii IFO 8158	−	−	nt	nt	−	−	nt	nt
Trichoderma harzianum IFO 31292	−	−	nt	nt	−	−	nt	nt
T. polysporum IFO 39322	−	−	nt	nt	−	−	nt	nt

The experiments were done in triplicate

−, No inhibition; ±, not clear inhibition zone; +, diameter of inhibitory zone <15 mm; ++, diameter of inhibitory zone 16–20 mm; +++, diameter of inhibitory zone 21–30 mm; nt, not tested

[a] Amount applied onto 8-mm I.D. paper disk

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