Purification and Characterization of Antifungal Substance Ac-1 Produced by a *Streptomyces* sp. AB-88M

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Key words: *Streptomyces* sp., antifungal antibiotic, Ac-1.

A microorganism which is antagonistic to various phytopathogenic fungi was isolated from the surface of a pear leaf in 1988 and designated as an isolate AB-88M⁴⁾. The morphological characteristics of this organism were the formation of spiral spore chains and sporangium-like bodies. The spiral spore chains and aerial mycelia intertwined and turned into a spherical sporangium-like body (3-10 μ m). By the scanning electron microscope (SEM) observation, the absence of the sporangial-wall was certified and so, this structure was decided as a pseudosporangium. These pseudosporangia fused each other forming a larger and irregular structure⁴⁾. This pseudosporangium released abundant spores at immersion in water. The spore was oval to spherical (0.6 $\times$ 0.7 μ m) and immobile with smooth surface. The analytical data of peptidoglycan layer⁸⁾ showed that the cell-wall type of this antagonistic microorganism is Type I (*Streptomyces* type). From whole morphological, biological and biochemical data^{4,5)}, this organism was identified as *Streptomyces* sp. Before 1986 this type of actinomycete had been grouped as *Actinosporangium* and this genus was recently transferred to the genus *Streptomyces*^{2,3)}. In this experiment the isolation of the antibiotic produced by this actinomycete was conducted. The abstract has been reported elsewhere⁹⁾ and the details will be contributed in this report.

The producer *Streptomyces* sp. AB-88M was inoculated on the PSA medium [1,000 ml of potato (200 g) decoction, 20 g sucrose, 18 g agar, pH 7.3] and the modified Bennett's medium [1 g yeast extract (Wako Co.), 1 g beef extract (Kyokuto Co.), 2 g casein hydrolysate (Sigma Co.), 10 g maltose, 1,000 ml dist. water, 18 g agar, pH 7.3] in petri dishes and incubated at 28°C for 2 days. The mycelial disk (5 mm in diameter) of the indicator fungus was confronted keeping distance 2 cm from the colony of the producer. Then, each petri dish was incubated at 28°C (20°C in the case of *Botrytis cinerea*) for 7 days and the antagonistic activity was estimated. The indicator fungi tested were as follows: *Pyricularia oryzae* Cavara (Naga 69-150), *Pyricularia* sp. (MZSV-1-1), *Botrytis cinerea* Persoon (85202R, 85299S), *Pestalotia funerea* Desmazieres (Ka-5), *Helminthosporium maydis* Nisikado et Miyake (NIAS-HM), *Helminthosporium oryzae* Miyabe et Hori (Kyu No. 10), *Rhizoctonia solani* Kühn (R57), *Alternaria kikuchiana* Tanaka (A4-5-2), *Fusarium roseum* f. sp. *cerealis* Snyder et Hansen (Kyu 78-13), *Fusarium solani* f. sp. *phaseoli* Snyder et Hansen (SUF386), *Fusarium oxysporum* f. sp. *melonis* Snyder et Hansen (KF320), *Sclerotium rolfsii* Saccardo (S2-1-2). The antagonistic activity was graded as : - not inhibited, + slightly inhibited, ++ inhibited, +++ strongly inhibited the mycelial growth of the indicator fungi.

Isolate AB-88M was shake-cultured with the modified Bennett's medium in Sakaguchi flasks at 30°C for 10 days. The culture fluid was centrifuged at 4,000 $\times g$ for 20 min. The supernatant obtained was shaken with equal volume of ethylacetate or chloroform in a separation funnel. The extract was concentrated *in vacuo* by a rotary evaporator at less than 40°C. After drying with N₂ gas blowing, the residue was dissolved in 90% aqueous methanol and subjected to the reverse phase Lobar RP-18 (Merck Co.) column and eluted with 90% aqueous methanol monitoring at 254 nm. The active fraction was diluted with twice volume of distilled water

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and shaken with a small volume of chloroform. The active substance was easily transferred to the chloroform and this fraction was subjected to silica gel thin layer chromatography (TLC) with two kinds of developing solvent such as chloroform-methanol (9:1 v/v, *R_f* 0.66) and benzene-acetone-methanol (28:7:4 v/v, *R_f* 0.45).

The reddish band on the plate was scraped and eluted with chloroform-methanol (2:1 v/v). The eluate was dried with N₂ gas blowing and dissolved in 80% aqueous methanol. Then, it was subjected to the reverse phase preparative high performance liquid chromatography (HPLC, Gilson Model 303) with C-18 M&S column (20×250 mm) and eluted with 80% aqueous methanol at flow rate 5 ml/min (Rt 23.5 min) monitoring at 313 nm. The active substance was transferred to a small volume of chloroform as stated before and recrystallized by the addition of *n*-hexane. Ultraviolet-visible light (UV/VIS) spectrum was obtained by the UV spectrophotometer (Hitachi Model 100-60) subjecting the methanol solution. Infra-red (IR) spectrum was obtained by using IR spectrophotometer (Jasco Model A-100) equipped with the beam condenser BC-2. To avoid a loss of the sample, KBr micro-disk method was used in the analysis. For the decision of molecular weight of this substance, the fast atom bombardment mass-spectrometry (FAB-MS(+)), JEOL JMS-DX300) and the low resolution electron impact ionization mass-spectrometry (LR-EIMS, JEOLJMS-300) were conducted. In the case of FAB-MS, chloroform-*n*-nitrobenzylalcohol was used as a solvent for analysis. The molecular formula was decided by the high resolution electron impact ionization mass-spectrometry (HR-EIMS, JEOL JMS-DX300) analysis.

The isolate AB-88M is antagonistic to various phytopathogenic fungi. As can be seen in Table 1, this organism inhibited the growth of most fungi tested. Especially, the inhibition was distinct against *Pyricularia*, *Botrytis*, *Pestalotia* and *Helminthosporium* spp. The antagonistic activity to *Fusarium roseum* f. sp. *cerealis* was also obvious, but not so severe against other *Fusarium* spp. Of the interest, this actinomycete was highly antagonistic to the causal agents of the air borne diseases and not so antagonistic against those of the soil borne diseases.

An antifungal antibiotic was extracted from culture filtrate easily by chloroform or ethylacetate and purified by TLCs, reverse phase LC & HPLC and recrystallization. This substance was dark-red amorphous substance and designated as Ac-1. The reddish color turned into bluish-purple by the spraying of 1 N NaOH solution or 0.25% methanol solution of magnesium acetate on the TLC plate. It has been well-documented that some *Streptomyces* spp. especially

Table 1. Antifungal activity of *Streptomyces* sp. AB-88M against various phytopathogenic fungi

Indicator fungi	PSA medium	Bennett's medium
<i>Pyricularia oryzae</i>	+++	+++
<i>Pyricularia</i> sp.	+++	+++
<i>Botrytis cinerea</i> (R)	+++	+++
(S)	+++	+++
<i>Pestalotia funerea</i>	+++	+++
<i>Helminthosporium maydis</i>	+++	+++
<i>Helminthosporium oryzae</i>	+++	+++
<i>Fusarium roseum</i> f. sp. <i>cerealis</i>	+++	+++
<i>Rhizoctonia solani</i>	++	++
<i>Alternaria kikuchiana</i>	++	++
<i>Fusarium solani</i> f. sp. <i>phaseoli</i>	+	+
<i>Fusarium oxysporum</i> f. sp. <i>melonis</i>	+	+
<i>Sclerotium rolfsii</i>	+	+

The producer *Streptomyces* sp. AB-88M was confronted against various indicator fungi on the PSA and Bennett's media.

(R): Resistant strain against thiophanate-methyl and benomyl.

(S): Susceptible strain against the chemicals stated above.

Fig. 1. Infra-red spectrum of antifungal substance Ac-1. KBr micro-disk was used.

Fig. 2. The positive ion FAB mass spectrometry of the antifungal substance Ac-1. Number 537 shows the value of $(M+H)^+$ and indicates the molecular weight of Ac-1 is 536. This value was certified by the results of LR, HR-EIMS. Ion source accel V: 2 kV, X_e^0 accel V: 5 keV. Solvent: $CHCl_3 + n$ -nitrobenzylalcohol.

the isolates which were formerly classified as *Actinosporangium* spp. produced various anthracycline antibiotics^{1,6,7}). UV/VIS spectrum of Ac-1 showed absorption peak at 210, 237, 313 and 509 nm and partly resembled with that of an anthracycline antibiotic produced by *Actinosporangium bohemicum*⁸). Such resemblance was also observed in the IR spectrum shown in Fig. 1. The molecular weight of Ac-1 was decided as MW 536 by the results of FAB-MS(+) and LR-EIMS (Fig. 2). Molecular formula was decided as $C_{34}H_{16}O_7$ by HR-EIMS analysis.

The species name of this *Streptomyces* and the chemical structure of this antibiotic Ac-1 will be decided in future study.

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和 文 摘 要

松山宣明： *Streptomyces* 属菌 AB-88M 株が産生する抗糸状菌物質 Ac-1 の精製とその諸性質

ナン葉葉上から分離された抗糸状菌物質産生性微生物 AB-88M 株は *Streptomyces* 属菌と同定された。本菌は *Pyricularia oryzae*, *Pyricularia* sp., *Botrytis cinerea*, *Pestalotia funerea*, *Helminthosporium maydis*, *Helminthosporium oryzae*, *Fusarium roseum* f. sp. *cerealis* に特に強い抗菌活性を示したが *Fusarium solani* f. sp. *phaseoli*, *Fusarium oxysporum* f. sp. *melonis*, *Sclerotium rolfsii* にはほとんど活性を示さなかった。本放線菌をベネット液体培地で 30°C, 10 日間振とう培養後、遠沈上清から活性物質を等量のクロロフォルムまたは酢酸エチルにより振出した。さらに2種の展開溶媒 (chloroform-methanol 9:1, benzene-acetone-methanol 28:7:4 v/v) によるシリカゲル薄層クロマトグラフィー、逆相系液体クロマトグラフィー (RP-18, 90% methanol), 分取型逆相系高速液体クロマトグラフィー (C-18, 80% methanol) および *n*-hexane による再結晶により精製し得られた暗赤色不定形結晶を Ac-1 と呼称した。各種質量分析の結果から、Ac-1 の分子量は、MW 536, 分子式は $C_{34}H_{46}O_7$ と決定された。

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