Isolation and structural properties of aerial mycelium differentiation-inhibitory substances against *Streptomyces scabiei* causing potato common scab

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The inhibitor of aerial mycelium differentiation in *Streptomyces scabiei*, a pathogen of potato common scab, was searched. An actinomycete isolated from the field was found to produce inhibitors and two active substances were isolated as single peaks with HPLC. The molecular weights of inhibitors A and B were 1156.5 and 1140.5, respectively. Numbers of functional groups in inhibitor A were estimated by derivatization reactions. Inhibitor A seems to be a non-peptidic or non-saccharic substance because enzymatic treatments with pepsin, trypsin or glycosidases did not affect the LC-MS profiles or inhibitory activity. © Pesticide Science Society of Japan

**Keywords**: potato common scab, *Streptomyces scabiei*, inhibitor, aerial mycelium, morphological differentiation.

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**Materials and Methods**

1. **Bacterial strains**

   The producer strain AO-4 was isolated from soil collected on Mt. Maehodaka, Nagano Prefecture, Japan. *Streptomyces* sp. B-9–1, the causative microorganism of root tumor of melon,10) was supplied by Prof. Masahiro Yoshida of Kyushu Tokai University, Japan.

2. **Bioassay**

   Inhibition of aerial mycelium differentiation was assayed by the paper disc method using *Streptomyces scabiei* S-851 provided by Dr. Nobuya Tashiro, Saga Fruit Tree Experiment Station11,12) as the test organism on oatmeal agar medium.13) After incubation at 28°C for 5 days, the diameters of the aerial mycelium-formation inhibitory zone and the growth inhibitory zone were measured.

3. **Enzymatic treatments**

   Treatments of inhibitor A with pepsin (Nacalai Tesque), trypsin (Sigma-Aldrich Japan) or glycosidase mixture (from *Turbor cornutu*, Seikagaku Corporation) were performed according to the methods reported by Ishizaki et al.14)

4. **Analytical methods**

   LC-ESI-MS was measured on a JEOL JMS-T100LC AccuTOF with the following conditions: column, TSKgel ODS-80Ts (2.0×150 mm; Tosoh); solvent system, CH₃CN–H₂O (6:4) with 0.1% CH₃CO₂H; flow rate, 0.2 ml/min; detection mode, ESI negative mode.

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**Results and Discussion**

1. **Screening of the producer of inhibitory substance**

   The production of phytotoxins is closely related to the differentiation of aerial mycelia from substrate mycelia, which is important as the preliminary step toward sporulation.9) When this differentiation is inhibited, spores will not be formed and spread of the disease will be suppressed, phytotoxin production will decrease and lesions will be reduced.

   We thus searched for an inhibitor of aerial mycelium differentiation of phytopathogenic actinomycetes such as *S. scabiei* and attempted to inhibit spore formation and phytotoxin production. In a previous report, we showed that some antibiotics could inhibit aerial mycelium formation and suppress phytotoxin production.9)

   Following our continued screening for a producer of aerial mycelium differentiation-inhibitory substances, we found an actinomycete tentatively named strain AO-4. We describe here the isolation and structural properties of the inhibitors produced by strain AO-4.

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was adopted as a bioassay because the dose-dependent effect of a sample can be observed at once\(^9\). Some examples are shown in Fig. 3. The culture filtrate of an unidentified actinomycete, strain AO-4, showed strong activity to inhibit aerial mycelium differen-

2. Isolation of the inhibitor

The productivity of the inhibitor in Hickey and Tresner’s medium gradually decreased. We thus examined other media and adopted Maltose-Bennett’s medium for mass cultivation.

Cultured material was treated as outlined in Fig. 1. In several preliminary experiments, comparable activity was observed in two fractions, i.e. 5% CH\(_2\)OH–CHCl\(_3\) and CH\(_2\)OH fractions, in silica gel column chromatography. The 5% CH\(_2\)OH–CHCl\(_3\) fraction, which showed similar activity with a lower dosage than the CH\(_2\)OH fraction, was then purified by ODS-silica gel column chromatography and activity was detected in two fractions, once again. Isolation of two major active substances from each active fraction was achieved by repeated preparative HPLC with CH\(_3\)CO\(_2\)H-containing solvent. While concentrating the HPLC eluate, azeotropic removal of acetic acid with toluene was indispensable to maintain activity. The yields of inhibitors A and B were 1.8 and 1.1 mg from 31 liters of cultured material, respectively. Inhibitors A and B induced an aerial mycelium-inhibitory zone of 1.9 cm diameter at 6 and 4 \(\mu g/disc\) (equivalent to 100 ml of cultured material), respectively.

3. Spectral analysis of inhibitors

The \(^1^H\)-NMR spectrum of inhibitor A (Fig. 2) shows that the purity was high and that it is a large molecule containing many hetero atoms. Inhibitor B showed a similar spectrum. We tried to measure the \(^1^C\)-NMR spectrum but a reliable number of carbon could not be determined since there was a limited amount of sample.

Inhibitors A and B showed the same UV spectra with \(\lambda_{\text{max}}\) 273 nm (\(\varepsilon\) 5550) and 222 nm (shoulder) in CH\(_2\)OH.

The mass spectrum of inhibitor A was obtained with LC-ESI-MS (negative mode), and a molecular-related ion ([M–H]\(^+\)) was observed at \(m/z\) 1155.5. Inhibitor B showed a peak at \(m/z\) 1139.5.

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**Fig. 1.** Purification procedure for aerial mycelium-formation inhibitors A and B from strain AO-4. Fractions with an underline are active fractions. Numerals under fractions denote: upper, yield; lower, diameter of aerial mycelium-inhibitory zone and that of the growth inhibitory zone in parentheses. Sample amounts for bioassay of filtrate and mycelial extract were those equivalent to 0.4 ml of cultured material and those of other fractions equivalent to 4 ml of that.

**Fig. 2.** \(^1^H\)-NMR spectrum of inhibitor A. The spectrum was measured in CD\(_3\)OD containing TMS as an internal standard at 600 MHz.
which was 16 mass units smaller than that of inhibitor A.

4. Derivatization of inhibitor A
To obtain information about the functional group, we prepared some derivatives of inhibitor A. Methylation with diazomethane increased the molecular weight by 56 mass units, indicating that inhibitor A has four carboxylic or phenolic groups. Acetylation with acetic anhydride in MeOH afforded two products with MWs of 1198.5 and 1240.5, which were 42 and 84 mass units larger than that of inhibitor A, respectively, indicating the presence of two amino or phenolic groups. Acetylation in dry pyridine also produced two products with MWs 1282.5 and 1324.5, implying that inhibitor A has a maximum of four acetylable sites. When combining the two results on acetylation, the presence of two alcoholic hydroxy groups was deduced.

5. Enzymatic treatment of inhibitor A
Inhibitor A was treated with pepsin, trypsin or a mixture of glycosidases, but no change was observed between LC-MS analyses of treated and non-treated samples (data not shown). The enzyme-treated samples maintained their inhibitory activity comparable with the non-treated sample in the bioassay. Under the same conditions, oligopeptide, bradykinin or steroidal saponin, digoxin was digested and the peak in the mass chromatogram disappeared with LC-MS analysis, indicating that the reaction conditions were appropriate. We could not detect any amino acid by LC-MS analysis of the acid hydrolyzate of inhibitor A. Thus, inhibitor A seems to be a non-peptidic or non-saccharic substance.

6. Speculation of molecular formula
From high-resolution MS analysis, many candidates were obtained and a reliable molecular formula could not be ascertained. We therefore refined the molecular formula with the intensities of isotope peaks of the molecular-related ion. From 10 high-resolution mass spectral data, the accurate mass and intensity ratios of isotope peaks were calculated as follows:

- accurate mass for \([M-H]^- : 1155.4902 \text{ mu} \pm 8.5 \text{ mmu}
- \([M-H+1]^-/[M-H]^- = 62.72 \pm 0.39\%
- \([M-H+2]^-/[M-H]^- = 22.71 \pm 1.11\%

In general, intensity ratios of isotope peaks for \(C_nH_{2n}N_{2n}O_{n}\) are shown by the following equations:\n
- \([M-H+1]^-/[M-H]^- = 1.1x + 0.36z
- \([M-H+2]^-/[M-H]^- = (1.1x)^2 + 0.20n\)

From these equations, the above data and the assumption that the number of nitrogen, \(z\), is less than 10, the numbers of \(C\) and \(O\) are estimated in the following ranges: \(53 \leq C \leq 57\), \(4 \leq O \leq 39\). Based on these considerations, eight probable molecular formulae were derived (candidates not shown), but a search in databases for these molecular formulae did not reveal any natural product. Some structures that were retrieved from the database had molecular formulae with one or two methylene units more or less than the speculated formulae or one oxygen less than the speculated formulae that corresponded to inhibitor B; however, none met the structural characteristics deduced above.

7. Effects of inhibitor A on other phytopathogenic Streptomyces species
Inhibitor A was effective on two \(S. scabiei\) strains (Table 1, Fig. 3 a, b) but did not show activity against two other pathogens of potato common scab, \(S. acidiscabies\) and \(S. turgidiscabies\). It also inhibited aerial mycelium formation associated with growth inhibition in \(S. ipomoeae\) sp. MAFF 225005, a pathogen of potato russet scab in Japan and in \(S. acidiscabies\) sp. B-9-1, that of root tumor of melon. In \(S. ipomoeae\), only distinct growth inhibition was observed (Fig. 3 d).

The first inhibitors of aerial mycelium formation, carbazomycinal and 6-methoxy carbazomycinal, were reported by Kondo et al.\(^\text{17}\) although the presence of such substances was previously indicated by McCann and Pogell.\(^\text{18}\) We showed that some antibiotics inhibiting protein biosynthesis suppressed the development of aerial mycelia and the production of phytoxins in \(S. scabiei\) and \(S. acidiscabies\).\(^\text{19}\) Two inhibitory substances reported here are large molecules and are non-peptidic or saccharic in character, indicating that they are a new type of inhibitor.

We are now investigating ways to improve the yield of these inhibitors, their partial degradation for structure determination and their effect on phytoxin production in phytopathogenic species.
**Streptomyces** spp. We are also continuing to search for inhibitors that will be lead to compounds for new agrochemicals.

**Table 1.** Effect of inhibitor A on phytopathogenic *Streptomyces* spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Aerial mycelium formation</th>
<th>Substrate mycelium growth</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. scabiei</em> S-851</td>
<td>1.7</td>
<td>–</td>
</tr>
<tr>
<td><em>S. scabiei</em> JCM 7914</td>
<td>1.5</td>
<td>–</td>
</tr>
<tr>
<td><em>S. acidiscabies</em> JCM 7913</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. turgidiscabies</em> NBRC 16080</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. ipomoeae</em> NBRC 14508</td>
<td>–</td>
<td>4.1</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. MAFF 225005</td>
<td>3.1</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Streptomyces</em> sp. B-9-1</td>
<td>3.4</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*a* Diameter (cm) of the inhibitory zone

**Notes:**
- *S. scabiei* S-851: 1.7 cm
- *S. scabiei* JCM 7914: 1.5 cm
- *S. acidiscabies* JCM 7913: –
- *S. turgidiscabies* NBRC 16080: –
- *S. ipomoeae* NBRC 14508: –
- *Streptomyces* sp. MAFF 225005: 3.1 cm
- *Streptomyces* sp. B-9-1: 3.4 cm

Sample amount was 6 μg/disc. – denotes no inhibitory zone was observed around paper disc (8 mm diameter).

**References**