Chemical and biological characterization of sclerosin, an antifungal lipopeptide

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Abstract: Pseudomonas sp. strain DF41 produces a lipopeptide, called sclerosin that inhibits the fungal pathogen Sclerotinia sclerotiorum. The aim of the current study was to deduce the chemical structure of this lipopeptide and further characterize its bioactivity. Mass spectrometry analysis determined the structure of sclerosin to be CH₃-(CH₂)₆-CH(OH)-CH₂-CO-Dhb-Pro-Ala-Leu/Ile-Ala-Val-Val-Dhb-Thr-Val-Leu/Ile-Dhp-Ala-Ala-Ala-Val-Dhb-Dhb-Ala-Dab-Ser-Val-OH, similar to corpeptins A and B of the tolaasin group, differing by only 3 amino acids in the peptide chain. Subjecting sclerosin to various ring opening procedures revealed no new ions, suggesting that this molecule is linear. As such, sclerosin represents a new member of the tolaasin lipopeptide group. Incubation of S. sclerotiorum ascospores and sclerotia in the presence of sclerosin inhibited the germination of both cell types. Sclerosin also exhibited antimicrobial activity against Bacillus species. Conversely, this lipopeptide demonstrated no zoosporicidal activity against the oomycete pathogen Phytophthora infestans. Next, we assessed the effect of DF41 and a lipopeptide-deficient mutant on the growth and development of Caenorhabditis elegans larvae. We discovered that sclerosin did not protect DF41 from ingestion by and degradation in the C. elegans digestive tract. However, another metabolite produced by this bacterium appeared to shorten the life-span of the nematode compared to C. elegans growing on Escherichia coli OP50.

Key words: lipopeptide, sclerosin, biocontrol, Pseudomonas, Sclerotinia sclerotiorum.

Résumé : Pseudomonas sp. souche DF41 produit un lipopeptide appelé sclérosine qui inhibe le pathogène fongique Sclerotinia sclerotiorum. Le but de cette étude était de déduire la structure chimique de ce lipopeptide et de caractériser davantage son activité biologique. Une analyse en spectrométrie de masse a permis de déterminer que la structure de la sclérosine était : CH₃-(CH₂)₆-CH(OH)-CH₂-CO-Dhb-Pro-Ala-Leu/Ile-Ala-Val-Val-Dhb-Thr-Val-Leu/Ile-Dhp-Ala-Ala-Ala-Val-Dhb-Dhb-Ala-Dab-Ser-Val-OH, similaire à celle des corpeptines A et B du groupe tolaasin, ne différant que par trois acides aminés de la chaîne peptidique. En soumettant la sclérosine à des procédés variés d’ouverture d’anneau, aucun nouvel ion n’a été détecté, suggérant que cette molécule est linéaire. Ainsi, la sclérosine représente un nouveau membre du groupe de lipopeptides tolaasin. L’incubation d’ascospores et de sclérotes de S. sclerotiorum en présence de sclérosine inhibait la germination des deux types de cellules. La sclérosine montrait aussi une activité antimicrobienne envers les espèces de Bacillus. À l’inverse, ce lipopeptide n’exerçait aucune activité zoosporicide envers le pathogène oomycète Phytophthora infestans. Par la suite, nous avons évalué l’effet de DF41 et d’un mutant dépourvu de lipopeptide sur la croissance et le développement de larves de Caenorhabditis elegans. Nous avons découvert que la sclérosine ne protégeait pas DF41 de l’ingestion par C. elegans et de la dégradation dans le tractus digestif. Cependant, un autre métabolite produit par cette bactérie semblait diminuer la longévité du nématode comparativement à C. elegans cultivé sur Escherichia coli OP50.

Mots-clés : lipopeptide, sclérosine, contrôle biologique, Pseudomonas, Sclerotinia sclerotiorum.

[Traduit par la Rédaction]

Introduction

Sclerotinia sclerotiorum is an economically important soil-borne pathogen capable of infecting over 400 plant species (Purdy 1979). In canola, S. sclerotiorum causes stem rot, and breeding for resistance has generally been unsuccessful, resulting in heavy reliance on fungicides for disease control. However, increasing concern over the use of agrochemicals and the coincident development of fungicide resistance has led to interest in alternative disease management strategies, including biological control. One such biocontrol agent, Pseudomonas species strain DF41 originally isolated from the canola rhizosphere, has demonstrated strong antagonism toward S. sclerotiorum (Berry et al. 2010; Savchuk and...
Lipopeptide molecules consist of a short peptide moiety linked to a fatty acid tail and are synthesized via a nonribosomal thiotemplate mechanism on peptide synthetases that utilize both standard and nonstandard amino acids to produce a broad range of lipopeptide products (Berti et al. 2007; Fiore et al. 2008). Sclerosin is a lipopeptide resembling members of the tolaasin group. In addition, it describes the influence of sclerosin on S. sclerotiorum ascospore and sclerotial germination, on the growth of Bacillus species, on the zoospores of the oomycete pathogen Phytophthora infestans, and on the growth and development of the nematode C. elegans.

**Materials and methods**

**Bacterial strains and growth conditions**

The bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli and Bacillus strains were cultured at 37 and 28 °C, respectively, on Lennox agar (Difco Laboratories). Pseudomonas species were cultured on King’s B (KB) medium at 28 °C or in M9CAlg (M9 minimal salts medium amended with 1% casamino acids (Difco) and 240 mmol/L glycerol). Antibiotics (Research Products International Corp.) were added to the media at the following concentrations: gentamicin (50 µg/mL), ampicillin (100 µg/mL), and kanamycin (50 µg/mL) for E. coli; tetracycline (15 µg/mL), piperacillin (80 µg/mL), rifampicin (50 µg/mL), kanamycin (5 µg/mL), gentamicin (40 µg/mL) for DF41.

**Mass spectrometry analysis of sclerosin**

Pseudomonas sp. strain DF41 and the sclerosin-deficient mutant DF41-1278 were grown in 900 mL volumes of M9CAlg medium for 4 days at 28 °C with shaking. Cells were removed by centrifugation (10 000 g for 10 min at room temperature), and metabolites, including sclerosin, were extracted from culture supernatants with ethyl acetate, dried, dissolved in methanol, and fractionated by high-pressure liquid chromatography (HPLC) (Berry et al. 2010). Fractions were analysed on a matrix-assisted laser desorption
ionization quadrupole-quadrupole-time-of-flight (MALDI QqTOF) instrument maintained by the Department of Physics at the University of Manitoba (Loboda et al. 2000). A 0.5 µL aliquot was mixed with an equal volume of saturated 2,5-di-hydroxybenzoic acid in water – acetonitrile (3:1 v/v) and 2% formic acid and were spotted onto the target. Spectra were collected in positive mode for 40 s. Spectra from matching fractions were compared to identify the ions unique to DF41, which were subjected to MS/MS on the same instrument (Shevchenko et al. 2000). Samples from DF41 HPLC fractionation were also analysed by electrospray ionization (ESI) on an ESI-TOF instrument built and maintained in the Department of Physics at the University of Manitoba (Kozlowski et al. 2005). ESI and MALDI spectra were analysed with TOPMA, an in-house software program.

Chemical treatments for lipopeptide hydrolysis
To determine if a lactone ring was present, aliquots of 100 µL were dried, dissolved in 100 µL of 0.5 mol/L ammonia in methanol, and incubated overnight at 37 °C to induce ring hydrolysis (Kuiper et al. 2004). Peptide fragments were generated by drying 100 µL aliquots of extract, dissolving in 100 µL of 25% trifluoroacetic acid (Zhong et al. 2005), and heating in a microwave oven for 5 min at high power. After heating, the extract was dried and resuspended in 5 µL of methanol before spotting onto the MALDI target. Selected ions were analysed by MS/MS for sequence determination. Parallel experiments were conducted using the cyclic lipopeptide surfactin (Sigma–Aldrich), for which MS data are available (Yang et al. 2006), to verify that the ring hydrolysis conditions were effective. All chemicals for MS were purchased from Fisher Scientific except trifluoroacetic acid, which was obtained from Pierce.

Sclerosin inhibits ascospore and sclerotial germination
The inhibitory effect of purified sclerosin on ascospore germination was determined by drying a 300 µL aliquot of the methanol extract containing sclerosin (methanol alone for the control) in a tissue culture dish and then dissolving it in 3 mL of molten potato dextrose agar (PDA, Difco). A sterile paper filter containing Sclerosin inhibits ascospore and sclerotial germination

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The effect of sclerosin on P. infestans zoospores
Phytophthora infestans US11 was maintained on V8-PDA agar (per litre: 150 mL V8 juice, 10 g PDA, 3 g CaCO3, 10 g bacto agar (Difco)). Zoospores were released from sporangia using the method described by Rohwer and colleagues (Rohwer et al. 1987). In brief, P. infestans was grown on V8-PDA for approximately 6 days until fungal hyphae covered approximately ¾ of a 35 mm × 10 mm Petri plate. The plate was then flooded with sterile water, and fungal hyphae were flattened using the bottom of a sterile glass test tube. The plates were dried and incubated overnight at room temperature to induce zoospore formation. Zoospores were harvested the next day by flooding the plate with sterile water. The suspension was removed from the plate and filtered through cheesecloth to eliminate any adherent mycelia. To release zoospores from sporangia, the filtrate was incubated at 4 °C for 2 h. A 20 µL aliquot of the zoospore suspension was added to a glass slide and microscopically observed for lysis upon addition of an equal volume of the purified lipopeptide.

Generation of DF41 and DF41-1278 expressing the mCherry red fluorescent protein
Derivatives of DF41 and DF41-1278 harbouring the mCherry red fluorescent protein (RFP) were created. To construct a plasmid that could be stably maintained in Pseudomonas, an 825 bp BamHI–EcoRI fragment containing the rfp gene from pRSET-B mCherry was cloned into the same sites of pUCP23. The resulting plasmid, pMCh-23, was then electroporated into DF41 and DF41-1278, creating DF41-rfp and DF41-1278-rfp. The optimal excitation and emission wavelength for mCherry RFP are 587 and 610 nm, respectively (Shaner et al. 2004).

Ingestion and digestion of DF41 and DF41-1278 by C. elegans
Caenorhabditis elegans laboratory strain Bristol N2 nematodes were maintained at 15 °C on nematode growth media spotted with benign live or UV-killed E. coli OP50 as a food source and were manipulated using established techniques (Hope 1999). To determine if LP41 causes any delay in C. elegans development from the larval stage to egg-laying adult stage, 5 larval stage 1 (L1) nematodes hatched on UV-killed E. coli OP50 were placed on lawns of DF41-rfp and DF41-1278-rfp. A 100 µL aliquot of an overnight bacterial culture grown in 1/10 KB was spotted onto a Petri plate (35 mm × 10 mm) containing 1/10 KB agar. The plates were incubated for 6 h at 28 °C to produce thin lawns of either DF41-rfp or DF41-1278-rfp. Assay plates were cooled to room temperature prior to seeding with C. elegans, followed by incubation at 25 °C for 2 weeks. For microscopic examination, nematodes were mounted on 2% agarose pads on glass microscope slides and anesthetized with 10 mmol/L Levamisole (Sigma–Aldrich) in M9 buffer. Nematodes were examined by Nomarski differential interference microscopy with an ApoTome-equipped Zeiss Axio Imager.

Antibacterial activity of DF41 and DF41-1278
The antibacterial activity of DF41 and DF41-1278 was tested using Bacillus megaterium, Bacillus thuringiensis, and Bacillus mycoides. Aliquots (5 µL) of DF41 and DF41-1278 grown in KB broth were spotted onto PDA plates and incubated for 48 h at 28 °C. Overlays containing 3 mL of an overnight Bacillus culture in 10 mL of 0.7% agar were added, and the plates were incubated at room temperature for 48 h before the zones of inhibition were measured.

Results and discussion
Sclerosin is structurally similar to the tolaasin group of lipopeptides
The structure of sclerosin was determined using MS. Preliminary MALDI-TOF MS analysis of secondary metabolites...
extracted from the growth medium of both DF41 and DF41-1278 revealed multiple components (Fig. 1), most of which were clustered at \( m/z < 1500 \), but 3 major ions at \( m/z 2095.3, 2123.3, \) and 2145.3 were unique to the DF41 extract. On HPLC fractionation, the ion at \( m/z 2095 \) eluted at 27 min, and the ions at \( m/z 2123 \) and 2145 both eluted at 29 min, apparently differing only by a sodium ion. Electrospray ionization of the samples from 27 and 29 min gave multiply charged ions of exactly the same mass as seen in the MALDI spectra (data not shown).

MALDI MS/MS applied to determine the amino acid sequences yielded similar patterns of fragments for the ions at \( m/z 2095, 2123, \) and 2145. Fragmentation in the MALDI QqTOF instrument produces mainly y and b ions (Biemann 1990) from which the sequence was manually assembled by “walking” along the spectrum (Fig. 2). A second b-type series (b*) lacking the N-terminal fatty acid and Dhb (253 Da smaller) was used to confirm the sequence, which was then tested using pTOOL. The absence of aromatic amino acids in the peptide portion was confirmed by the lack of absorbance in the 260–280 nm range. Because the \( m/z 2095 \) and \( m/z 2123 \) and 2145 ions yield the same peptide sequence, the 28 Da difference lies in the fatty acid chains — 3-hydroxyoctanoic acid in 2095 and 3-hydroxydecanoic acid in 2123 and 2145 — consistent with the difference in retention times on the HPLC. The sequence determined for the 2123 and 2145 ions is \( \text{CH}_3-(\text{CH}_2)_6-\text{CH(OH)}-\text{CH}_2-\text{CO-Dhb-Pro-Ala-Leu/Val-Met-Val-Dhb-Thr-Leu/Ile-Dhp-Ala-Ala-Ala-Val-Dhb-Dhb-Ala-Dab-Ser-Val-OH} \).

While cyclic lipopeptides are common, a linear sclerosin was suggested by the clear identification of ions in the region where cyclization would be expected. The linear nature was confirmed by the lack of new ions appearing after treatment with ammonium–methanol solution (Kuiper et al. 2004; Nutkins et al. 1991) or heating in 25% trifluoroacetic acid. As a control, the cyclic lipopeptide surfactin yielded C-terminus ions with an additional 18.01 Da when treated in the same way (Yang et al. 2006). Thus the 3 ions \( m/z 2095 \) and 2123 and 2145 are most likely linear.

Strain DF41-1278 contains a Tn5 insertion in a region with high sequence identity to an enzyme involved in the synthesis of syringopeptins (Berry et al. 2010), a class of lipopeptides in the tolaasin group (Raaijmakers et al. 2006). Like sclerosin, this group of lipopeptides contains a high proportion of hydrophobic as well as unusual amino acids within an 18–25 amino acid peptide portion. In particular, the ions in sclerosin are similar in size and sequence to those of corpeptin A (\( m/z 2094 \)) and corpeptin B (\( m/z 2120 \)), 2 lipopeptides from the tolaasin group, produced by \( Pseudomonas corrugata \) that exhibit phytotoxic activity (Emanuele et al. 1998). Indeed, the peptide sequence of sclerosin differs from corpeptins A and B by only 3 amino acids, which are shown
in boldface and are underlined (3-hydroxydecanoic acid—Dhb) in Table 2.

Table 2. Observed ions from the MS/MS (tandem mass spectrometry) spectrum shown in Fig. 2 compared with those predicted (expected) by pTOOL.

<table>
<thead>
<tr>
<th>Ion</th>
<th>m/z</th>
<th>Predicted residue</th>
</tr>
</thead>
<tbody>
<tr>
<td>b0</td>
<td>NO (1.0)</td>
<td>—</td>
</tr>
<tr>
<td>b1</td>
<td>NO (171.1)</td>
<td>—</td>
</tr>
<tr>
<td>b2</td>
<td>254.2 (254.2)</td>
<td>b0 NO (1.0)</td>
</tr>
<tr>
<td>b3</td>
<td>351.2 (351.2)</td>
<td>b1 NO (98.1)</td>
</tr>
<tr>
<td>b4</td>
<td>422.2 (422.3)</td>
<td>b2 169.1 (169.1)</td>
</tr>
<tr>
<td>b5</td>
<td>535.3 (535.4)</td>
<td>b3 282.2 (282.2)</td>
</tr>
<tr>
<td>b6</td>
<td>606.4 (606.4)</td>
<td>b4 353.2 (353.2)</td>
</tr>
<tr>
<td>b7</td>
<td>705.4 (705.5)</td>
<td>b5 452.3 (452.3)</td>
</tr>
<tr>
<td>b8</td>
<td>804.5 (804.5)</td>
<td>b6 551.3 (551.4)</td>
</tr>
<tr>
<td>b9</td>
<td>887.5 (887.6)</td>
<td>b7 634.4 (634.4)</td>
</tr>
<tr>
<td>b10</td>
<td>988.6 (988.6)</td>
<td>b8 735.4 (735.4)</td>
</tr>
<tr>
<td>b11</td>
<td>1087.6 (1087.7)</td>
<td>b9 834.5 (834.5)</td>
</tr>
<tr>
<td>b12</td>
<td>1200.7 (1200.8)</td>
<td>b10 947.6 (947.6)</td>
</tr>
<tr>
<td>b13</td>
<td>NO (1269.8)</td>
<td>b11 1016.6 (1016.6)</td>
</tr>
<tr>
<td>b14</td>
<td>1340.7 (1340.8)</td>
<td>b12 1087.6 (1087.7)</td>
</tr>
<tr>
<td>b15</td>
<td>1411.8 (1411.9)</td>
<td>b13 1158.7 (1158.7)</td>
</tr>
<tr>
<td>b16</td>
<td>1482.9 (1482.9)</td>
<td>b14 1229.7 (1229.7)</td>
</tr>
<tr>
<td>b17</td>
<td>1581.9 (1582.0)</td>
<td>b15 1328.7 (1328.8)</td>
</tr>
<tr>
<td>b18</td>
<td>NO (1665.0)</td>
<td>b16 1411.8 (1411.8)</td>
</tr>
<tr>
<td>b19</td>
<td>NO (1748.0)</td>
<td>b17 1494.8 (1494.9)</td>
</tr>
<tr>
<td>b20</td>
<td>NO (1819.1)</td>
<td>b18 1585.9 (1586.0)</td>
</tr>
<tr>
<td>b21</td>
<td>1919.1 (1919.1)</td>
<td>b19 1665.9 (1666.0)</td>
</tr>
<tr>
<td>b22</td>
<td>2006.1 (2006.2)</td>
<td>b20 1753.0 (1753.0)</td>
</tr>
<tr>
<td>b23</td>
<td>2105.0 (2105.2)</td>
<td>b21 1852.0 (1852.1)</td>
</tr>
</tbody>
</table>

Note: NO, not observed.
* ions lack the 253 Da of the N-terminal fatty acid and Dhb.

Sclerosin inhibits S. sclerotiorum ascospore and sclerotal germination

To further characterize the inhibitory effect of sclerosin on the growth of S. sclerotiorum, ascospores and sclerotalia were treated with the methanol extracts from DF41 (containing sclerosin) and DF41-1278 (no sclerosin) (Fig. 3). Both ascospore and sclerotal germination were completely inhibited by the sclerosin-containing extract but were unaffected by the extract of DF41-1278. That sclerosin is strongly antagonistic to the fungal pathogen S. sclerotiorum sets it apart from other members of the tolaasin group of lipopeptides, which mainly exhibit phytotoxic activity, except for tolaasin itself, which is toxic to mushrooms. Clearly, sclerosin is not phytotoxic, at least to canola plants (Berry et al. 2010), and the effective inhibition of ascospore and mycelial germination suggests the mechanism by which DF41 suppresses S. sclerotiorum proliferation.

DF41 displays antibacterial activity against Bacillus species

Antibacterial activities have been ascribed to lipopeptides and several members of the tolaasin group, particularly the syringopeptins, that inhibit the growth of B. megaterium (Emanuele et al. 1998; Grgrurina et al. 2005; Lavermicocca et al. 1997). Consistent with inclusion of sclerosin in the tolaasin group, DF41, but not DF41-1278, displayed strong inhibition of B. megaterium and slightly less antagonism towards B. thuringiensis and B. mycoides (Table 3).

Sclerosin does not affect zoospores of the oomycete pathogen P. infestans

The zoospore membranes of P. infestans, an oomycete pathogen causing late blight in potato and tomato plants

Table 3. Antimicrobial activity of DF41 and the sclerosin-deficient DF41-1278 against Bacillus species.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Zone of inhibition (mm)</th>
<th>P. syringae pv. syringae B728a</th>
<th>P. syringae pv. syringae B728a</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. megaterium</td>
<td>DF41 9.0 (1.5)</td>
<td>DF41-1278 9.0 (1.0)</td>
<td>9.0 (1.0)</td>
</tr>
<tr>
<td>B. thuringiensis</td>
<td>DF41 4.0 (2.0)</td>
<td>DF41-1278 0.0 (0.0)</td>
<td>4.5 (1.5)</td>
</tr>
<tr>
<td>B. mycoides</td>
<td>DF41 6.0 (3.0)</td>
<td>DF41-1278 0.0 (0.0)</td>
<td>12.5 (2.0)</td>
</tr>
</tbody>
</table>

Note: Values are the mean (standard deviation) obtained from 6 replicates.
Sclerosin-mediated inhibition of *Sclerotinia sclerotiorum* ascospore and sclerotia germination. Filter papers containing ascospores were applied to potato dextrose agar (PDA) alone (A), PDA containing sclerosin extract (B), or PDA containing dried methanol (C). Plates were examined after 72 h at room temperature. Arrows point to ascospores that have germinated (panels A and C) or have not germinated (panel B). Sclerotia that were untreated (D) or pre-treated with sclerosin (E) or with methanol (F) were placed onto PDA and allowed to incubate at room temperature for 72 h.

In summary, *Pseudomonas* sp. strain DF41 produces the lipopeptide sclerosin that closely resembles corpeptins, members of the tolaasin class of lipopeptides, and represents a new addition to this class. The biological activity of sclerosin differs in presenting a strong antifungal activity against *S. sclerotiorum* as well as antibacterial activity against *Bacillus* species. On the other hand, sclerosin is not phytotoxic towards canola plants, shows no inhibition of *P. infestans* oomycete growth, and does not affect grazing by or passage through *C. elegans*.

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