Viscosin, a Potent Peptidolipid Biosurfactant and Phytopathogenic Mediator Produced by a Pectolytic Strain of *Pseudomonas fluorescens*†

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The structure of a peptidolipid isolated from a pectolytic strain of *Pseudomonas fluorescens* biovar II has been determined by using mass spectrometry, NMR spectroscopy, and chemical methods. The compound was identified as viscosin, an antiviral peptidolipid first isolated from *P. viscosa*. The native peptidolipid is a potent surfactant that facilitates bacterial infection and the spread of decay on nonwounded broccoli florets colonized by these pathogenic bacteria.

INTRODUCTION

Head rot is a destructive disease that accounts for significant losses of broccoli grown in Atlantic Canada and in many other regions of the world. Recently, pectolytic strains of *Pseudomonas fluorescens* biovars II and IV (also referred to as *P. marginalis*) have been identified as the primary pathogens responsible for broccoli head rot (Hildebrand, 1989; Wimalajeewa et al., 1987). Apart from their ability to produce tissue-macerating enzymes, a key factor in their ability to cause decay is the production of extracellular surfactant material(s) (Hildebrand, 1989). Reduction of the surface tension of liquid media inoculated with these strains has indicated biosurfactant production (unpublished data). Evidence of altered surface activity by the bacteria can also be observed on host tissues, including the appearance of water-soaked areas and black lesions around stomata on the otherwise waxy surface of the broccoli florets. Guard cells and surrounding cells of stomata in affected areas also become discolored, indicating bacterial invasion. Following this early colonization phase, the tissues become soft and a rapidly spreading decay ensues with continued wetness.

The hypothesis of a surfactant-mediated mechanism of decay was strengthened when the pectolytic strains of *Erwinia carotovora* var. carotovora and *E. carotovora* var. atroseptica were found to be incapable of causing decay of broccoli except when tissues were wounded (Hildebrand, 1989). However, when these strains were coinoculated with nonpectolytic but surfactant-positive saprophytic strains of *P. fluorescens*, rapid decay followed. The symptomology of this decay was similar to that caused by pathogenic strains of *P. fluorescens* bearing both pectolytic and surfactant attributes.

A variety of microorganisms produce potent surfactants, and the subject of microbial biosurfactants has been reviewed by different authors (Zajic and Panchal, 1976; Zajic and Seffens, 1984). The most active biosurfactant discovered to date is surfactin, a peptidolipid produced by *Bacillus subtilis* capable of reducing the surface tension to water to 27 mN/m (Cooper and Zajic, 1980). Pseudomonads are also known producers of surfactant, and some of these compounds have been isolated and characterized. Rhamnolipids have been isolated from *Pseudomonas aeruginosa* (Edwards and Hayashi, 1965; Itoh et al., 1971), and an ornithine lipid was found in extracellular fluids of *Pseudomonas rubescens* (Wilkinson, 1972). Recently, a high molecular weight surfactant of unknown structure was found in extracellular fluids of *P. fluorescens* biovar I (Persson et al., 1988).

To understand the host–parasite relationship between broccoli and surfactant-producing strains of *P. fluorescens* and to develop disease-control measures, we describe herein the overall strategy and methods applied to isolate this phytopathogenic agent and to characterize its complete structure by chemical analysis, mass spectrometry, and NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Bacterial Growth Conditions. A bacterial isolate designated SH10-3B was obtained from decayed broccoli and identified as a pectolytic strain of *P. fluorescens* biovar II (Hildebrand, 1989). The isolate was maintained in sterile distilled water at room temperature and subcultured on *Pseudomonas* agar F (PAF, Difco Laboratories). The isolate was cultured in a synthetic liquid medium similar to that of Brocklehurst and Lund (1981) but without any yeast extract, casamino acids, cysteine hydrochloride, and tryptophan as these were not essential for biosurfactant production. The medium contained (grams per liter) glucose (10.0), NH_4Cl (1.0), $MgSO_4 \cdot 7H_2O$ (0.2), KH_2PO_4–K_2PO_4 buffer, pH 6.8 (0.05 M), and trace metals as (milligrams per liter) $CaCl_2$ (15.0), $FeSO_4 \cdot 7H_2O$ (10.0), $CuSO_4 \cdot 5H_2O$ (2.0); $ZnSO_4 \cdot 7H_2O$ (2.0); $MnSO_4 \cdot H_2O$ (1.5), $CoCl_2 \cdot 6H_2O$ (0.2), and Na_2MoO_4 (0.2). Isotopically labeled surfactant was obtained by replacing NH_4Cl with $^{15}NH_4Cl$. For inoculum production a loopful of bacteria, obtained from 48-h-old growth on PAF, was transferred to 100 mL of liquid medium and subsequently incubated at 22 °C for 18 h. Two hundred milliliters of liquid medium was then inoculated with 1.0 mL of the inoculum and incubated on a shaker at 24 °C for 5 days. Alternatively, the bacterial isolate was cultured on plates (9-cm diameter) of PAF medium. Forty plates were each inoculated with 0.2 mL of a heavy bacterial suspension and incubated at 22 °C for 4 days, after which time the bacterial growth was gently washed with distilled water from plates and combined to a volume of 300 mL.

The isolate was also grown on broccoli tissues. A heavy bacterial suspension was obtained by washing growth from 72-h-old plates of PAF, and cells were then centrifuged and resuspended in sterile distilled water three times to remove any existing surfactant. Small pieces of nonabsorbent cotton (1.0 cm^3) were then soaked in a suspension of washed cells and placed on several broccoli heads that had been wounded by making numerous shallow incisions on the florets with a scalpel. The heads were then placed in a moist chamber at 24 °C for 6 days. The chamber

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was equipped with a misting apparatus to provide continuous moisture. Approximately 860 g of decayed tissue that developed was then scraped from the heads and agitated in 1200 mL of water. The mixture was then filtered through several layers of cheesecloth and the filtrate retained for analysis.

Extraction of Biosurfactant. Bacterial cell suspensions obtained from the synthetic liquid medium cultures on PAF or from decayed tissues of broccoli were centrifuged at 20000*g* for 30 min. The cell-free supernatant was acidified to pH 2 with 2 N HCl and allowed to stand at 2 °C for at least 1 h. The white precipitate that formed upon acidification was centrifuged at 20000*g* for 15 min, the supernatant was decanted, and the pellets were extracted with 60 mL of 95% ethanol. Undissolved material was filtered off and reextracted in ethanol. The residue was discarded, and the two ethanolic extracts were combined and concentrated on a rotary evaporator to 10–15 mL. A small amount of activated charcoal was added to remove any color, and the solution was then filtered through Celite. The surfactant crystallized overnight after water was added until the ethanolic solution was slightly turbid. The colorless needle-shaped crystals were washed with cold water.

Surface Activity Measurements. Surface tension, interfacial tension, and the critical micelle concentration (cmc) were measured by using a tensiometer (Cenco Instruments, Chicago) with a du Nouy platinum ring (ascending method). Interfacial tension was measured by submerging the ring in an aqueous solution of the surfactant and adding sufficient hexadecane so that the ring did not break the surface of the hexadecane before pulling through the interfacial film (Akit et al., 1981). The cmc was determined by measuring the surface tension of successive dilutions of known quantities of surfactant in distilled water and plotting the surface tension against the logarithm of concentration. The point of the graph at which the surface tension increased sharply was recorded as the cmc.

Amino Acid Analyses. Weighed samples of dried, crystalline surfactant were hydrolyzed in 6 N HCl for 24 h at 110 °C in sealed, evacuated tubes. Hydrolysates were evaporated to dryness and redissolved in water and amino acid concentrations determined by using a Beckman 119-CL amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA). Absolute configurations of amino acids were established by amino acid analyses after deamidation of acid-hydrolyzed surfactant with D- or L-amino acid oxidase (Sigma, St. Louis).

HPLC Separations. The recrystallized ethanolic extract was injected into a Vydac 218TP52 C_{18} analytical column (2.1 cm × 25 cm) of 5-μm particle size (Separations Group, Hesperia). A gradient elution varying from 50:50 to 100:0 acetonitrile/water in 20 min, and with 0.1% trifluoroacetic acid, was performed on a HP 1090 (Hewlett-Packard, Fort Collins, CO) operating at 0.2 mL/min, with a diode array detector set for single-wavelength detection at 214 nm. Preparative HPLC was performed on the same system using a Vydac 218TP510 column (10 cm × 25 cm) at a flow rate of 4.2 mL/min.

NMR Spectroscopy. Most NMR spectra were obtained with a Bruker MSL-300 spectrometer at 300.13 (^{1}H), 75.5 (^{13}C), or 30.4 MHz (^{15}N), using near-saturated solutions in methanol-d_4 in 5-mm tubes, reference internal TMS, temperature 20 °C. Additional ^{1}H spectra were acquired at 500 MHz with a Bruker AM-500 spectrometer to resolve overlap. The following types of spectra were obtained [see, e.g., Derome (1987) for descriptions of methods]: surfactant at natural isotopic abundance, conventional ^{1}H, ^{1}H COSY, ^{13}C {^{1}H}-broadband-decoupled, ^{13}C coupled, ^{13}C-DEPT, ^{13}C–^{1}H heterocorrelation optimized for 1-bond couplings; ^{15}N-labeled surfactant, conventional ^{1}H, ^{13}C {^{1}H}-broadband-decoupled, ^{15}N-coupled, and {^{1}H}-broadband-decoupled with suppression of NOE.

Mass Spectrometry. GC–MS analyses were conducted on a Finnigan 4500 quadrupole mass spectrometer equipped with a Finnigan 9610 gas chromatograph and an INCOS data system. Extracts were injected (1 μL) directly on a fused silica capillary column (25 m × 0.32 mm) wall coated with DB5-30W (Chromatographic Specialities Inc., Brockville, Canada). Helium (flow rate 1.5 mL/min, linear velocity 30 cm/min) was used as carrier gas. Oven temperature was programmed from 60 to 300 °C at a rate of 10 °C/min and kept at final temperature for 15 min. The mass spectrometer was operated either in electron impact (EI) mode (70 eV) or in chemical ionization (CI) mode using isobutane as reagent gas. A VG Analytical ZAB-EQ (VG Analytical, Manchester, U.K.) hybrid tandem mass spectrometer equipped with a VG 11-250J data system was used in all +LSIMS and MS–MS experiments. Approximately 1 μg of the purified biosurfactant was deposited on a gold-tipped probe and dissolved in 1 μL of 2-hydroxyethyl disulfide/thioglycerol (2-HEDS/THIO). A 30-keV Cs^+ beam (VG Analytical) was used. Conventional mass spectra were obtained by scanning the magnet over a mass range of 50–1300 Da in 10 s with a static resolution of 1500 (10% valley definition). Manual peak matching at a static resolution of 5000 against known standard (cesium iodide) was performed to establish the accurate mass of the protonated molecular ions. Precision of these measurements was typically ±10 ppm. In MS–MS analysis the decelerated/focused precursor ion beam from the double-focus point was transmitted into the RF-only quadrupole collision cell. Collisional activation spectra were obtained at 40–50 eV (laboratory reference frame) by using argon as collision gas at pressures such that the precursor ion intensity was attenuated by 90% of its original value. The fragments formed in the collision cell were analyzed by scanning the final quadrupole. All MS–MS data were acquired in MCA mode, each spectrum representing the accumulation of at least nine scans.

RESULTS AND DISCUSSION

The surface tension of the liquid medium inoculated with isolate SH10-3B dropped rapidly (within 25 h of inoculation) to a minimum value of 25 mN/m, and surfactant content continued to increase thereafter. A similar surface tension was observed in solutions obtained by washing cells from PAF. Almost complete recovery of biosurfactants from the medium was achieved by acidifying the cell-free supernatant and extracting the precipitate with ethanol. A colorless, crystalline product was then obtained from the concentrated ethanolic solution by adding cold distilled water. Long, thin, white needles, mp 230 °C, were formed upon standing overnight at 2 °C; these were washed with water and dried over P_2O_5 at room temperature. Approximately 1 g of precipitable surfactants was obtained either from 67 plates (9-cm diameter) or from 6 L of liquid medium.

The surface tension of a saturated solution of the purified extracellular product in water was 27 mN/m. Approximately 4–9 mg/L was required to reach the cmc, and the interfacial tension against hexadecane was 11.4 mN/m. This material can accumulate in the medium to concentrations (up to 500 mg/L) much greater than its solubility in water (10 mg/L). This phenomenon cannot be attributed to micelle formation but appears to be due to binding of surfactants to acidic polysaccharides. A gel was visible around bacterial colonies on agar plates, and this material was freely soluble in liquid culture media. High molecular weight acidic polysaccharides are known to be produced by plant-associated fluorescent pseudomonads (Fett et al., 1989), and the exopolysaccharide from *P. marginalis* was shown to consist of a 1,3-linked galactoglucan with pyruvate and succinate substituents (Osman and Fett, 1989).

The procedure we have adopted relies on coprecipitation with acidic polysaccharides which flocculate on acidification. Calcium ions or salting out from 50% saturated ammonium sulfate solutions may also be used to precipitate the polysaccharide/peptidolipid complex. A coprecipitation method was also used to isolate surfactin, a peptidolipid produced by *B. subtilis* (Arima et al., 1968). The high molecular weight biosurfactant from strain 378 of *P. fluorescens* (Persson et al., 1988) was possibly a polysaccharide–surfactant complex as well, but a lipopeptide component was not separated.

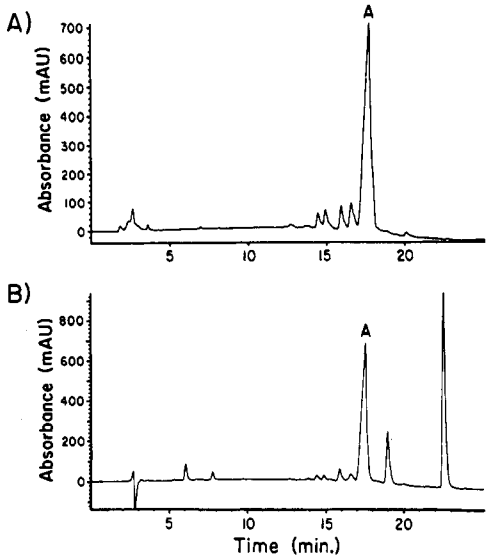
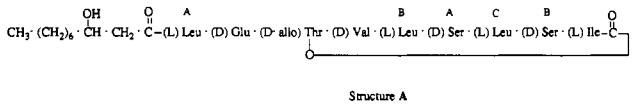


Figure 1. HPLC profile (at 214 nm) of bacterial metabolites from *P. fluorescens* biovar II. Analysis of 5 μg of extracellular biosurfactants from growth of the organism of PAF (A) and on broccoli florets (B).

Profiles of Extracellular Biosurfactants. The crystalline extract isolated from cultures of SH10-3B on PAF, synthetic liquid medium, or on the broccoli host were analyzed by HPLC (214 nm). The surface-active metabolites started to elute from the reverse-phase C_{18} analytical column at 75–80% acetonitrile and were completely eluted at 100% acetonitrile. A typical HPLC trace of biosurfactant metabolites isolated from the PAF cultures (Figure 1A) shows a major component eluting at 17.5 min. A similar HPLC profile was also obtained for cultures of SH10-3B on synthetic medium (data not shown). The result of the HPLC analysis performed on the broccoli surfactant extract (Figure 1B) was different from those of the in vitro cultures, showing additional peaks at 19.0 and 22.5 min. All of these samples, however, contained a major metabolite eluting at 17.5 min (conveniently labeled A). Similar chromatographic conditions applied on a preparative HPLC scale yielded fractions of individual components. In liquid cultures compound A



Structure A

accounted for approximately 90% of the total weight of the sample, while for cultures on PAF this peak accounted for 70%. Recrystallization of A from aqueous ethanol gave colorless long, thin needles, mp 270–273 °C. Careful recrystallization of the crude surfactant (mp 230 °C) from aqueous ethanol resulted in a purer product with the same melting point as the HPLC-purified compound A.

Surface tension measurements of A obtained in both water and aqueous KH_2PO_4–K_2HPO_4 (pH 7) were 27.0 and 25.0 mN/m, respectively. The difference between these measurements is probably due to increased solubility of the compound in the presence of phosphate and the effect of electrolytes on surfactants (Attwood and Florence, 1983. It is noteworthy that surface tension measurements of this material in the synthetic liquid medium were the same as obtained with the phosphate buffer. Plots of the logarithm of concentration against surface tension were similar for purified compound A and for the cell-free

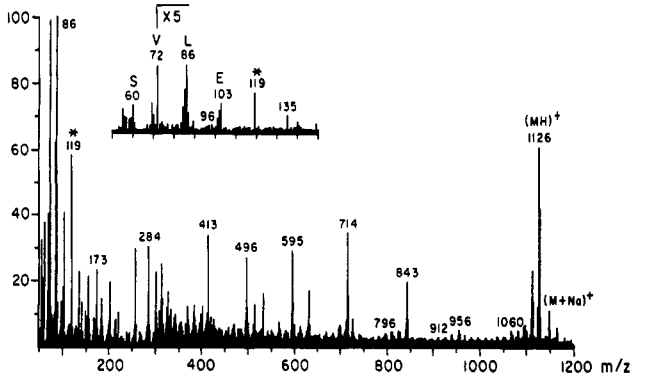


Figure 2. +LSIMS mass spectrum of approximately 2 μg of compound A. One-letter code is used to designate immonium ion of the corresponding amino acid. Asterisks indicate matrix ions.

supernatant extracts from plates or synthetic liquid cultures. The cmc in the extract from the liquid medium and the purified surfactant was approximately 10 mg/L. However, higher cmc values (up to 100 mg/L) were obtained when the surfactant was not dissolved completely upon sonication. The fact that A is the major component found in extracellular extracts of isolate SH10-3B, coupled with the corresponding surface tension measurement, provided strong evidence to suggest that most of the surface activity found in these cultures can be accounted for by this compound or its polysaccharide complex in the medium.

Structural Characterization. The molecular weight of A was determined by mass spectrometry using + LSIMS desorption ionization (Figure 2) and thermogravimetric analyses. Intense ion signals were observed at m/z 1126, 1148, and 1164 Da (Figure 2), which were assigned to the protonated molecular ion and to the adducts of sodium and potassium, respectively. Accurate mass measurement performed by using peak matching established that the monoisotopic component of the protonated species has a mass of 1126.71 ± 0.01 Da. The elemental analysis (C, 56.93%; H, 8.64%; O, 23.30%; N, 11.15%) was consistent with the empirical formula $C_{54}H_{97}O_{17}N_9$ (molecular weight, 1144 Da), assuming that only nine nitrogen atoms are present (see following discussion on amino acid analysis). The difference of 18 mass units between values obtained by mass spectrometry and elemental analysis is attributable to water of crystallization. The empirical formula of the peptidolipid would then be $C_{54}H_{95}O_{16}N_9$, which is consistent with the exact mass measurement.

Although A did not react with ninhydrin, possibly due to steric hindrance or unavailability of any free amine functionality, six amino acids were released by acid hydrolysis. Their corresponding molar ratios were as follows: Allo-Thr (0.79), Ser (2.06), Glu (1.05), Ile (1.10), Leu (3.11), and Val (1.05). The amino acid analysis of the hydrolysate showed no detectable Allo-Thr, Ser, or Val after a 2-h reaction with D-amino acid oxidase. Molar ratios for Leu, Glu, and Ile were unchanged. Thus, only Allo-Thr, Ser, and Val were in the D configuration, while the other amino acids are L isomers. Experiments with L-amino acid oxidase confirmed these findings. Glu was identified as the D isomer by isolation from an acid hydrolysate and direct determination of its optical rotation in a polarimeter, $[\alpha]^{23}_D$ −24° (c 0.26 in 0.1 M HCl). A negative optical rotation for Thr was consistent with the D-allo isomer. From the proposed empirical formula, $C_{54}H_{95}O_{16}N_9$, the amino acid content ($C_{44}H_{77}O_{14}N_9$) accounts for 85% of the molecular weight of the biosurfactant A, while the remaining $C_{10}H_{18}O_2$ indicated a hydroxydecanoic acid moiety.

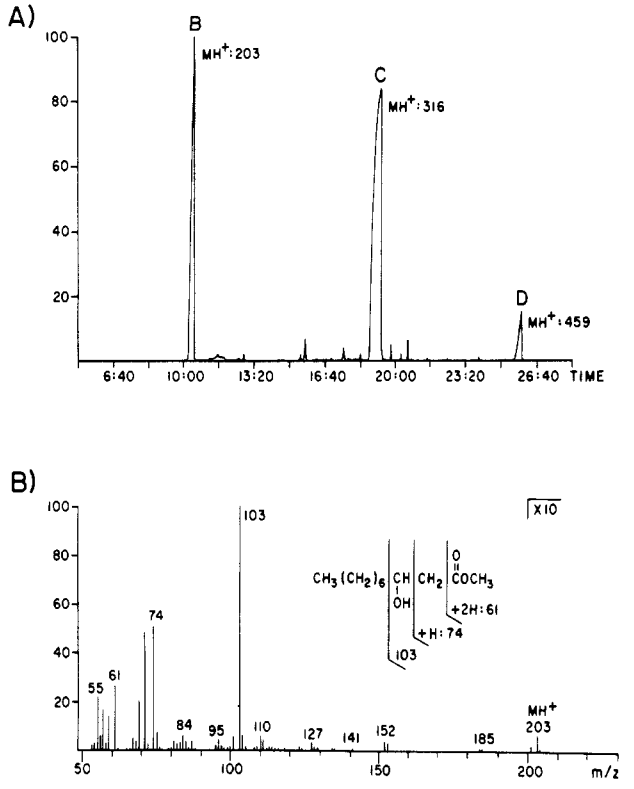


Figure 3. GC–EIMS analysis of hexane-soluble extract from methanolysis. (A) Total ion chromatogram from EI ionization; protonated molecular ion originating from self-protonation are indicated by MH^+. (B) Conventional EI mass spectrum of compound B.

The acid hydrolysis left a water-insoluble solid that was methylated by diazomethane and the hexane extract subjected to GC–EIMS analysis without further purification. The total ion chromatogram obtained under electron ionization (Figure 3A) indicated three major components whose mass spectra were dominated by low mass fragments and weak molecular ion intensities. Confirmation of the molecular weight of the three methylated residues was obtained by repeating the same analysis under chemical ionization (CI) conditions with isobutane and showed that the three compounds had protonated molecular ions of 203, 316, and 459 Da, respectively. Compound B (Figure 3) corresponds to the methylated fatty acid, C and D correspond to the fatty acid combined with a methylated leucine and with a leucylglutamyl methyl ester, respectively. This information is consistent with A containing a fatty acid covalently bonded to the N-terminal function of the peptide via an amide bond to a (Leu-Glu) moiety.

The EI mass spectrum of the methyl ester derivative of the fatty acid is illustrated in Figure 3B. A protonated molecular ion is observed at m/z 203. Although it would be expected that a molecular ion should be observed in EI, this result is consistent with self-protonation occurring within the ionization source (Harrison, 1983). Numerous low-mass fragments of structural importance were clearly distinguished at 61, 71, 74, 84, 95, 103, and 110 Da. Comparison of this spectrum with a mass spectral library (Mass Spectral Library, 1974) gave a best fit for 3-hydroxydecanoic acid methyl ester. The location of the hydroxyl group was indicated by the base peak at m/z 103, the ion arising through an α-cleavage at the far side of the hydroxyl group (Ohdam and Stenhagen, 1972). This assignment was confirmed by the observation of fragment ions at m/z 74 and 71, which correspond to losses of CHO and methanol from the ion at 103, respectively, and by NMR spectroscopy (described later).

Further structural information on A was deduced from fragment ions observed in the mass spectra of both the natural and methylated biosurfactant. Following treatment with diazomethane, the conventional mass spectrum of the methylated derivative (data not shown) displayed a protonated molecular ion 14 Da higher than the corresponding molecular ion of the naturally occurring biosurfactant. This indicated the presence of only one free carboxyl group. The amino acid content obtained from the hydrolysate analysis of A was confirmed by the observation of characteristic immonium ions $(NH_2{=}CH{-}R)^+$ appearing in the low mass region (m/z < 160 Da) of the +LSIMS mass spectrum (Biemann and Martin, 1987; Kulik and Heerma, 1988). In the case of the underivatized peptidolipid (Figure 2) these ions were observed at m/z 60, 72, 86, and 102, corresponding respectively to immonium ions of serine, valine, leucine (or isoleucine), and glutamic acid. The immonium fragment for glutamic acid was not observed in the methylated biosurfactant, but instead an intense ion was observed at 116 Da. This is consistent with a methyl ester glutamyl immonium ion and suggests that glutamic acid is the only free carboxylic functionality in the peptidolipid. On the basis of the above information and the double-bond equivalent, it can be deduced that the peptidolipid contains one ring structure.

MS–MS experiments established the connectivities between fragment ions and provided sequence assignment of both the native and methylated peptidolipids. Daughter ion spectra of all the major sequence ions of A (Figure 2) were recorded to establish the sources and sinks of these different species. Major sequence ions were regrouped in two types, viz., fragments containing both lipid and amino acids and those for which the charge is located on the C-terminal segment formed only of amino acid residues. The daughter ion spectra of examples of each of these two types are shown in parts A and B of Figure 4 for fragment ions 284 and 843 Da as precursors.

Rationalization of the fragment ions observed in the MS–MS spectra was facilitated by the prior knowledge of the lactone–ester bond location obtained from NMR spectroscopic data (described in more detail in the following discussion). The low chemical shift observed on the β proton of the threonine side chain (δ_H 5.48 compared to a value of δ 4.22 for a "random coil" threonine residue; Wüthrich, 1976) suggested that cyclization occurred at the threonine residue. As the hydroxyl group of the fatty acid is not involved in the ester bond, this indicated that a linear segment containing at least the 3-hydroxydecanoate and leucylglutamyl residues is linked to the cyclic peptidolipid. This conclusion was supported by the observation of strong fragment intensities at 284 and 413 Da (Figure 2) and their respective counterfragments at 843 and 714 (Figure 4) for which the positive charge is located on the C-terminal portion of the peptidolipid. For the methylated peptidolipid fragments, 413 and 843 Da were both shifted by 14 Da, confirming the position of the glutamic acid.

Fragment ions observed in the MS–MS experiments were rationalized by considering that protonation is localized at the lactone group. Cleavage of an ester bond results in the formation of an acylium ion or a protonated C-terminal carboxylic acid (Biemann and Martin, 1987; Das et al., 1979). Subsequent fragmentation occurring at the peptidic bond via simple cleavage or hydrogen transfer gave rise to fragment ions indicated in Figure 4B and were indicative of the amino acid sequence Glu-Thr-Val-Leu-

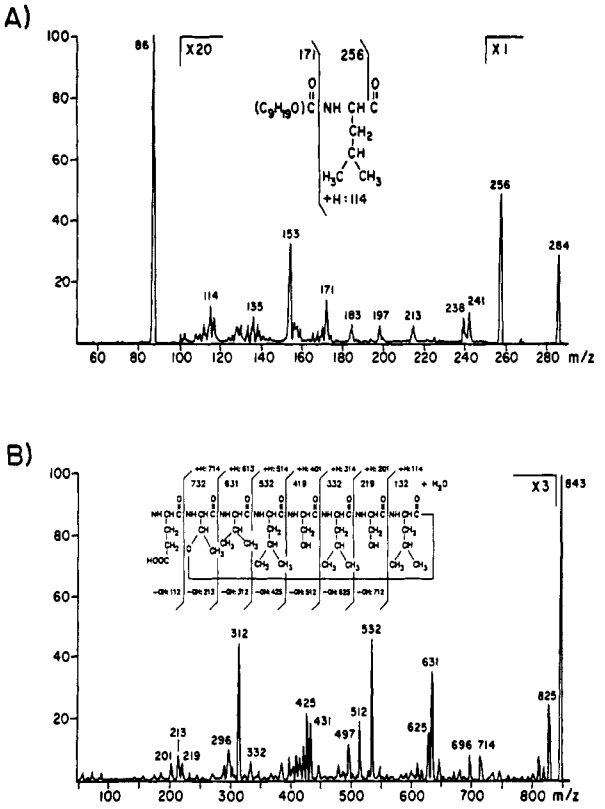


Figure 4. Collision-induced dissociation spectrum of daughter ions from m/z 284 Da (A) and 843 Da (B) of compound A. Argon target gas 90% attenuation; E_{lab} = 30 eV.

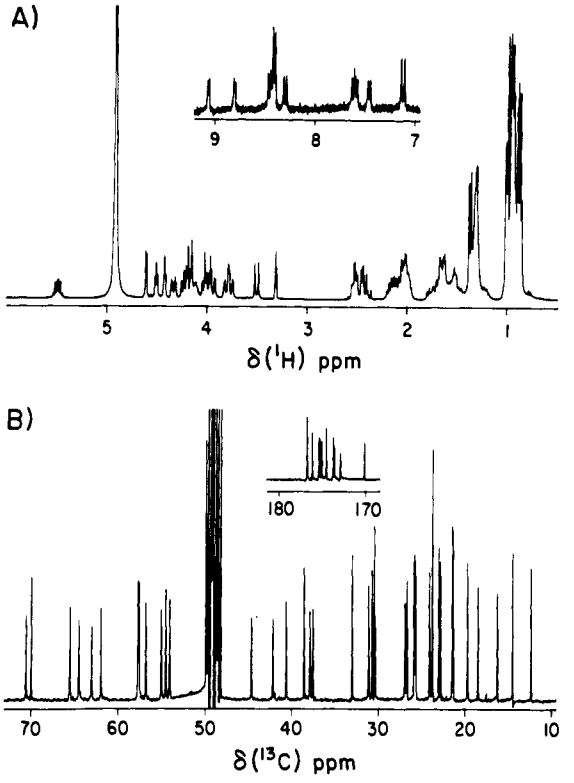


Figure 5. 300-MHz ^1H (A) and 75.5-MHz ^{13}C (B) NMR spectra of compound A. Solvent, methanol-d_4; reference, TMS. ^1H resonances at partially exchanged sites are shown on an expanded vertical scale (insert).

Ser-Leu-Ser-Leu. It should be noted that the isoleucine (as distinct from leucine) location was not yet firmly confirmed by the above mass spectrometric analyses and that its assignment was made on the basis of NMR data.

Assignments in the ^1H and ^{13}C NMR spectra of the purified material (Figure 5A,B) were made from 2D COSY and heterocorrelation spectra. Resonances of each amino acid residue were identified by starting with an α carbon resonance in the region δ_C 54–71, locating the resonance of the α proton via the heterocorrelation spectrum, and using the COSY spectrum to find ^1H peaks of the side chain. Correlations to amide protons were visible when exchange with the solvent was incomplete. The ^{13}C resonances of each side chain was then located with the heterocorrelation spectrum, which also resolved overlapping proton resonances. The characteristic COSY coupling patterns of the side chains were consistent with the information obtained from the amino acid and mass spectrometric analyses. All side-chain resonances were assigned individually apart from those of the Leu methyl groups, which could not be distinguished from each other due to overlap of the methyl proton resonances. Chemical shifts for both ^{13}C and ^1H, along with the ^{13}C–^{15}N coupling constants observed, are reported in Tables I and II. With the exception of Thr, as discussed previously, these shifts were in agreement with reported values for amino acid residues (Wüthrich, 1976, 1986), making allowance for differences in solvent.

The connectivity of the remaining resonances proved that they corresponded to a 10-carbon fatty acid. ^{13}C chemical shifts for the chain were in close agreement with predicted values based on assignments for 3-aminodecanoic acid (Sadtler and Standard ^{13}C spectra) and substituent effects for replacement of NH_2 by OH and COOH by $CONR_2$ (Levy et al., 1980). The OH substitution at the 3-position was confirmed by the observation of a ^{13}C chemical shift of 70.0 ppm of this CH group. The ^1H of this CH group was coupled with two adjacent CH_2 groups at δ_C 44.6, δ_H 2.43 and at δ_C 38.55, δ_H 1.53 (Tables I and II). The former CH_2 group showed no further coupling, indicating that it was next to a CO, whereas coupling from the latter CH_2 group could be traced to its neighbor and from there to the center of the saturated chain.

Experiments with the ^{15}N-labeled compound established the location of the lactone–ester linkage and the nature of the residue bonded to the Thr β carbon (which from the MS–MS spectrum could have been either Leu or Ile). Infrared spectroscopy also provided definite evidence of a lactone–ester link (band at 1742–1748 cm^{-1}). Lactone ester cyclization was confirmed by the observation of a doublet corresponding to an α carbon resonance of Ile at δ_C 57.64, due to ^{13}C–^{15}N coupling to the Ile ^{15}N only. All other α carbon resonances showed an additional coupling via the CO group to the ^{15}N of the adjacent residue. Thus, Ile is the residue connected by a lactone–ester link to the Thr β carbon. Further proof that the δ_C 57.64 resonance corresponds to an Ile α carbon followed from the fact that the resonance of the directly bonded ^1H (δ_H 4.60) was a doublet when the solvent had completely exchanged all NH protons for deuterium. The resonances of all Leu α protons were doublets of doublets. A lactone–ester link between Ile and Thr accounted for the unusually low shift of the Thr β proton (5.48 ppm) compared to the normal random coil value of 4.22 ppm (Wüthrich, 1986), which suggests acylation at this position, and was confirmed by single-frequency ^1H decoupling ($\gamma H_2/2\pi$ ca. 300 Hz), which demonstrated that the Thr β ^1H was coupled to the Ile carbonyl ^{13}C.

The side chain of Glu was proven to be a free acid, as linkage of this residue via the amide CO to the adjacent Thr was confirmed by the ^{13}C resonances of the Glu α carbon (δ_C 56.80), which was a doublet of doublets due to

Table I. ^{13}C Chemical Shifts (ppm) and Coupling Constants (J) for Viscosin[a]

residue	C_α	C_β	C_γ	C_δ	CO	$J_{C,N}$, Hz	$J_{CO,N}$, Hz
Leu (A)	55.00	42.15	25.80 or 25.85	21.3 and 23.0 or 23.7 or 24.1	175.06 or 175.24 or 175.37[b]	10.5 6.6	14.9 or 14.4 or 13.9
Leu (B)	54.49	40.60	25.67	21.3 and 23.0 or 23.7	as for Leu (A)	10.6 7.6	as for Leu (A)
Leu (C)	54.04	37.86	25.80 or 25.85	21.3 and 23.0 or 23.7 or 24.1	as for Leu (A)	10.1 6.8	as for Leu (A)
Ile	57.64	37.53	26.65 16.24	12.34	170.10[c]	10.6	no coupling
Ser (A)	57.70	63.05			170.60 or 173.72[b]	11 ± 2 7 ± 2	15.8 or 15.3
Ser (B)	57.56	64.52			as for Ser (A)	13 ± 2 7 ± 2	as for Ser (A)
Val	65.55	30.34	19.69 22.80		174.51[b]	9.7 6.6	14.0
Glu	56.80	25.62	31.07	176.13[c]	176.74[b]	10.2 6.6	14.6
Thr	61.96	70.62	18.47		172.88[b]	10.6 7.5	16.2
3-OH-decanoic moiety (C_1–C_{10})	176.74 ($J_{C,N}$ = 14.6), 44.64 ($J_{C,N}$ = 7.0), 70.01, 38.55, 26.87, 30.43, 30.70, 33.00, 23.73, 14.48						

[a] Solvent CD_3OD; reference TMS. [b] Tentatively assigned by comparison with reported values for amino acids residues (Wüthrich, 1976). [c] Confirmed by single-frequency decoupling or by ^{13}C–^{15}N coupling.

Table II. ^1H Chemical Shifts (ppm) for Amino Acid Residues and the Fatty Acid Moiety of Viscosin[a]

residue	H_α	H_β	H_γ	H_δ	NH
Leu (A)	4.35	1.63 2.01	1.6	0.84–1.0 0.9–1.0	7.60
Leu (B)	4.02	1.63 1.78	1.77	1.0 1.0	8.8
Leu (C)	3.82	1.68 2.01	1.6	0.84–1.0 0.9–1.0	8.41
Ile	4.60	2.01	1.32, 1.62, 0.89	0.90	7.13
Ser (A)	4.50	3.79 3.94			8.30
Ser (B)	4.41	3.97 4.17			7.62
Val	3.51	2.18	0.96		7.43
Glu	4.23	2.07	2.51		9.06
Thr	4.17	5.48	1.37		8.45
3-OH-decanoic moiety (H_2-2′–H_3-10′)	2.43, 4.12, 1.53, 1.37, 1.32, 1.31, 1.30, 1.32, 0.90				

[a] Solvent CD_3OD; reference TMS.

^{13}C–^{15}N coupling with both the Glu and Thr ^{15}N nuclei, whereas the Glu γ carbon resonance was a singlet. Alternatively, linkage via the γ carbon of Glu would have resulted in a doublet for each of the Glu α and γ ^{13}C resonances due to the coupling with a single ^{15}N nucleus. Linkage of the fatty acid chain to the N-terminal Leu was also proven, as the resonance of the $^{13}CH_2$ group (δ_C 44.64, C-2′) was a doublet due to ^{13}C–^{15}N coupling (Table I), and N^1H resonance were present for each amino acid residue.

The above information is consistent with structure A for the surfactant peptidolipid. This structure corresponds to that of viscosin, an antibiotic cyclodepsipeptide originally isolated from *P. viscosa* (Kochi et al., 1951). The original structure of viscosin published in 1953 (Ohno et al., 1953) was revised in 1970 (Hiramoto et al., 1970), and its synthesis was reported recently (Burke et al., 1989). Apart from the present findings, knowledge of the surface activity of viscosin is quite recent. Studies conducted independently (Neu et al., 1990) have also shown that viscosin will reduce the surface tension of water to 27 mN/m. Interestingly, this compound was also purified from a strain of *P. fluorescens* while screening for bacteria with hydrophobic surfaces. It is possible that the high molecular weight complex isolated from a strain of *P. fluorescens* biovar I by Persson et al. (1988) may also contain viscosin on the basis of their surface tension measurement of 27 mN/m. It would be interesting to determine if viscosin production is a taxonomic trait among all biovars of *P. fluorescens*.

The surfactant phenomenon in the host–parasite relationship of broccoli head rot is novel and of interest to plant pathologists, as the phytopathological role of an extracellular surfactant has not been previously reported. On the basis of observed symptoms of the disease, it is likely that viscosin acts to overcome the interfacial surface tension imparted by the host epicuticular waxes. This would enable penetration of the cuticle and stomata by pectolytic enzymes to sites of degradation in the cell walls. Physical spreading of bacteria-laden droplets on the broccoli head is also an important function of viscosin in the infection process. A nonpectolytic strain of *P. fluorescens* biovar II also yielded viscosin (data not shown), which indicates the potential of saprophytic strains to interact with pectolytic, surfactant negative strains as previously noted (Hildebrand, 1989). Thus, viscosin may play a significant role in other host–parasite or saprophytic ecological relationships. Bunster et al. (1989) have also recently observed increased wettability of wheat leaves following colonization by strains by *P. fluorescens* and *P. putida*. Although surface tensions of growth media were only moderately affected by these strains, growth in other media may result in greater surface activity (Bunster et al., 1989).

The surface activity of viscosin, and its relative ease of production and extraction demonstrated in this study and that of Neu et al. (1990), indicates the potential for practical applications of this product as a biosurfactant (Cooper, 1986). While the present paper is mainly concerned with the characterization of the major metabolite isolated from *P. fluorescens* biovar II, studies are presently being conducted on the same organism to characterize the structures and properties of other extracellular biosurfactants. These results will be reported separately. In addition to its surfactant properties, viscosin was originally described as an antibiotic showing activity against mycobacteria and bronchitis virus of chickens, as well as slight but detectable activity against infection of influenza A virus in mice (Groupé et al., 1951).

The ability of viscosin to cause leakage of nutrients from plant tissues indicates a disruptive effect on cell mem-

branes, and it is likely that its antibiotic properties also result from the potent surface activity of this compound. While no critical comparison of the properties of viscosin with other biosurfactants presently exists, it appears that viscosin is at least as effective as surfactin (Cooper, 1986) in its ability to lower surface tension of water. The pure compound can be prepared quite cheaply and could find applications as an industrial biosurfactant. Alternatively, the cell-free culture medium containing the viscosin complex may also find practical applications where a cheap surfactant is needed as, for example, in certain crop sprays.

ABBREVIATIONS USED

PAF, *Pseudomonas* agar F; cmc, critical micelle concentration; HPLC, high-performance liquid chromatography; GC–EIMS, gas chromatography–electron impact mass spectrometry; GC–CIMS, gas chromatography–chemical ionization mass spectrometry; +LSIMS, positive ion liquid secondary ion mass spectrometry; MS–MS, tandem mass spectrometry; 2-HEDS/THIO, 2-hydroxyethyl disulfide/thioglycerol (1:1); MCA, multiple-channel acquisition; ^{1}H NMR, proton nuclear magnetic resonance; ^{13}C NMR, carbon nuclear magnetic resonance.

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