Isolation and Partial Chemical Characterization of an Antimicrobial Peptide Produced by a Strain of *Bacillus subtilis*†

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An antimicrobial lipopeptide has been isolated, purified to homogeneity, and partially characterized from a strain of *Bacillus subtilis* found in apple fruit. The compound consists of an acidic peptide containing aspartic acid, glutamic acid, serine, glycine, alanine, proline, valine, and leucine in a ratio of 2:3:1:1:1:1:1:4. The infrared spectrum indicates that the peptide antibiotic contains an acyl chain and has a lactone bond in its structure. On the basis of amino acid analysis, sodium dodecyl sulfate−polyacrylamide gel electrophoresis, and gel filtration data, the molecule has a molecular mass of 1.5 kDa but also forms aggregates in excess of 20 kDa. The peptide antibiotic appears to be similar to a number of lipopeptide antibiotics often termed "biosurfactants" and classified as fatty acid-containing peptolides. The antibiotic demonstrates a broad spectrum of activity against Gram-negative bacteria, shows little activity against Gram-positive organisms, and is active against one of the two fungi assayed.

Keywords: *Antibiotic; lipopeptide; lactone; endophytic; biosurfactant*

INTRODUCTION

Many types of antibiotics are produced by a wide assortment of microorganisms. Over 8000 antibiotics are known to exist, and hundreds more are discovered yearly (Brock and Madigan, 1991); however, few prove to be commercially useful. With the concern that pathogenic bacteria are quickly becoming resistant to commonly used therapeutic agents, the search for new antibiotics is becoming increasingly important. According to Brock and Madigan (1991), antibiotics are produced in excess of 100 000 tons per year with annual gross sales amounting to nearly $5 billion.

Frequently, a number of chemically related antibiotics exist such that they can be arranged into chemical classes or families; peptide antibiotics represent such a family. Peptide antibiotics are a very diverse group of compounds in terms of form and function. According to Katz and Demain (1977) the peptide antibiotics are best described not by a single definition, but rather by a number of properties: they are smaller than most proteins; generally a family of closely related peptides rather than a single substance is produced by an organism; they often contain constituents other than amino acids; they often contain unique amino acids; and they are often cyclic. These properties are often used to differentiate peptide antibiotics from proteins and other classes of antibiotics.

In the search for new peptide antibiotics, the genus *Bacillus* is an excellent place to look. *Bacillus* species produce a large number of peptide antibiotics representing at least 25 different basic chemical structures (von Döhren, 1995). Of all the antibiotics produced by *Bacillus* spp., most are active against Gram-positive organisms; however, compounds such as polymyxin, colistin, and circulin exhibit activity against Gram-negative organisms (Katz and Demain, 1977). Some of the peptides produced by *Bacillus* species, mainly the lipopeptides, demonstrate antifungal properties (Galvez et al., 1993). Examples include iturins (Delcambe and Devignat, 1957), bacillomycins (Peypoux et al., 1981), and mycosubtilins (Besson and Michel, 1990). Interestingly, some of the peptide antibiotics produced by *Bacillus* spp. not only demonstrate antimicrobial properties but are excellent surfactants. Jenny et al. (1991) suggest that surfactin, a lipopeptide antibiotic, is the most effective biosurfactant discovered so far.

The chemical and physical diversity of peptide antibiotics makes them ideal candidates not only for therapeutic applications but also in other areas, especially the agri-food industry. In some cases, peptide antibiotics that demonstrate surface active properties (biosurfactants) may even be useful to the petroleum industry (Fiechter, 1992).

From a study aimed at using endophytic *Bacillus* species isolated from stored apples to control postharvest diseases (Sholberg et al., 1995), one of the bacterial isolates was selected for further studies. A cell-free culture supernatant of the bacterial isolate demonstrated marked antibacterial and antifungal activity, suggesting that the bacteria produces some type of extracellular inhibitory compound. The isolation, purification, and partial characterization of this antimicrobial agent are presented in this paper.

MATERIALS AND METHODS

Microorganisms and Culture Conditions. The antibiotic producing organism (designated EN 63-1) was isolated from the internal tissue of Golden Delicious apples taken from

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cold storage in Oliver, BC. and determined to be *Bacillus subtilis* (Sholberg et al., 1995). The antibiotic indicator organism, *Agrobacterium vitis* CG 1005, was generously supplied by T. Burr, New York State Agricultural Station, Geneva, NY. Bacteria stocks were stored at −70 °C in 20% glycerol. When necessary, a loopful of either *B. subtilis* or *A. vitis* was transferred from frozen stock to 20 mL of tryptic soy broth (Difco Laboratories, Detroit, MI) or nutrient broth (Difco), respectively, in a 150 mL cotton-plugged Erlenmeyer flask.

A. vitis and *B. subtilis* were incubated at 27 and 30 °C, respectively, in an incubator shaker (Environ-Shaker 3697-PR, Lab-Line Instruments Inc., Melrose Park, IL) operated at 150 rpm.

Production of the antibiotic was achieved in a minimal defined medium derived from McKeen et al. (1986). The medium contained 20 g of dextrose, 5 g of DL-glutamic acid, 1.02 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of K_2HPO_4, 0.5 g of KCl, and 1 mL of trace element solution per liter of distilled water. The trace element solution consisted of 0.5 g of $MnSO_4 \cdot H_2O$, 0.16 g of $CuSO_4 \cdot 5H_2O$, and 0.015 g of $FeSO_4 \cdot 7H_2O$ per 100 mL of distilled water. One hundred milliliters in each of two 500 mL Erlenmeyer flasks was seeded with 1 mL of *B. subtilis* taken from frozen stock. The flasks were incubated for 32 h in the above incubator shaker maintained at 30 °C and 150 rpm.

Antibiotic Assay. Liquid samples containing the antibiotic were assayed for activity using an agar-well diffusion assay (Tagg and McGiven, 1971). Fifty microliters of an *A. vitis* liquid culture (frozen stock) was spread onto the surface of a 60 by 15 mm Petri dish containing 8 mL of potato dextrose agar (Difco) using a sterile bent glass rod. A well was made in the center of the plate using a No. 3 cork borer, and a 50 μL antibiotic sample was pipetted into the well. The sample was allowed to diffuse into the agar, and the plate was inverted and incubated at 27 °C until a lawn of the indicator bacteria appeared on the plate.

To determine the antibiotic titer, a sample was applied to the agar-well diffusion assay at 1-, 5-, 10-, and 20-fold dilutions. The titer of the sample was calculated as the inverse of the sample dilution giving an inhibition of 20 mm in diameter. The result was expressed as activity units per milliliter (AU/mL).

Microbial Spectrum. The following 10 bacterial and 2 fungal species, respectively, were tested for their sensitivity to the antibiotic using the agar-well diffusion assay: *Escherichia coli*, *Salmonella typhimurium*, *Erwinia amylovora*, *A. vitis*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas corrugata*, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Penicillium expansum*, and *Botrytis cinerea*. Aliquots of an overnight culture of the bacteria being assayed for sensitivity to the antibiotic were added to sterile 1% (w/v) peptone water blanks to yield a bacterial suspension with a final optical density (600 nm) of 0.3. Fifty microliters of the bacterial suspension was spread onto an agar plate and used in the agar-well diffusion assay as previously described for *A. vitis*. The two fungal strains assayed were tested in the same manner; however, spore suspensions (3.0×10^5 spores/mL) were used in the assay. Spore suspensions were made by transferring spores from a 7-day-old culture to a distilled water blank using a sterile cotton swab. Spore suspensions were adjusted using a cell counting chamber (Dynatech, Germany).

Purification of the Antibiotic. A bulk purification of the antibiotic was achieved by precipitating it from the culture supernatant according to the method of McKeen et al. (1986). Concentrated HCl was added to 50 mL of cell-free culture supernatant until a pH of 2.0 was obtained. The resulting precipitate was recovered by centrifuging the mixture at 15000*g* for 10 min (model J2-21M, Beckman Industries Inc., Palo Alto, CA). The supernatant was discarded, and 5 mL of 50 mM tris, pH 7.5, was added to the pellet. The pellet was dislodged from the bottom of the centrifuge tube and dissolved in the tris buffer using the pestle from a tissue homogenizer. The pH of the solution was adjusted to 7.5 with 0.1 M HCl, and the solution was filtered through No. 4 filter paper (Whatman Inc., Clifton, NJ). The purification was carried out at room temperature to this point.

The crude extract (5 mL) was applied to a 1.5 by 15 cm Bio-Rad low-pressure chromatography column (Bio-Rad Laboratories, Hercules, CA) filled with DEAE-Sephacel (Pharmacia, Uppsala, Sweden) equilibrated in 50 mM Tris-HCl, pH 7.5. A gradient of 0−0.4 M NaCl in buffer (50 mM, pH 7.5) was run through the column at 0.5 mL/min over a period of 160 min followed by straight 0.4 M NaCl in buffer (50 mM, pH 7.5) for 40 min. The eluent was monitored at 280 nm, and fractions were collected every 5 min. An Econo System low-pressure chromatography unit (Bio-Rad) was used to make the gradient, monitor the eluent, and collect the fractions. Ion exchange chromatography was performed at 4 °C, and all samples were assayed for activity using the agar-well diffusion assay.

Liquid−liquid extraction, adapted from the method of Morikawa et al. (1993), was used to recover the antibiotic from the ion exchange eluent. The active fractions from ion exchange chromatography were pooled and shaken vigorously in a 50 mL capped centrifuge tube with 5 mL of reagent grade isobutanol at room temperature. The resulting emulsion was centrifuged at 15000*g* for 10 min in a Beckman model J2-21M centrifuge. The upper isobutanol phase was carefully drawn off the aqueous phase, and the aqueous phase was discarded. The isobutanol was evaporated to dryness under a stream of nitrogen gas. The resulting residue of semipurified antibiotic was used for most of the analyses.

Further purification was achieved using an HPLC protocol modified from that of Hozono and Suzuki (1983). Approximately 1.5 mg of the partially purified antibiotic was weighed out and dissolved in 0.5 mL of acetonitrile/1% acetic acid (68:32). Fifty microliters was loaded onto a 5 μm (4.0 × 250 mm) Super Pac cartridge system Pep-SC_2 C_{18} reversed-phase column (Pharmacia) equilibrated with acetonitrile/1% acetic acid (68:32). The antibiotic was eluted from the column using the same buffer at a flow rate of 1.0 mL/min. The eluent was monitored at 220 and 280 nm with a Waters 990 photodiode array detector (Waters Associates, Milford, MA). Solvent delivery was achieved with a Waters 510 solvent delivery system. Each peak from the chromatogram was manually collected and tested for activity using the agar diffusion assay.

Ultraviolet Spectrum. The ultraviolet absorbance spectrum of the antibiotic was measured from the HPLC chromatogram using a Waters 990 photodiode array detector. This device allowed for the simultaneous measurement at all wavelengths from 200 to 600 nm as the antibiotic eluted from the column.

Infrared Spectrum. The infrared spectrum of the antibiotic was measured as a potassium bromide pellet. Three hundred micrograms of semipurified antibiotic was mixed with 250 mg of KBr and ground to a fine powder. Approximately one-third of the KBr mixture was pressed into a pellet (8 mm diameter) using a non-evacuable Mini Press (Perkin-Elmer, Norwalk, CT), and four scans of the sample were taken using a Perkin-Elmer 1600 FTIR spectrophotometer.

Amino Acid Analysis. HPLC-purified antibiotic was used for amino acid analysis. Ten microliters of the HPLC-purified antibiotic was applied to an amino acid analyzer (model 420A derivatizer and model 130A separation system, Applied Biosystems, San Francisco, CA). The sample was hydrolyzed in 6 M HCl at 200 °C for 24 h in a sealed ampule and derivatized with phenylisothiocyanate prior to separation on a Brownlee 5 μm (2.1 × 20 mm) C_{18} reversed-phase column.

SDS−Polyacrylamide Gel Electrophoresis (PAGE). Gel electrophoresis was performed according to the method of Schagger and von Jagow (1987). Tricine−SDS−PAGE gels were made and run using a Mini Protean II slab gel electrophoresis unit (Bio-Rad). One millimeter spacers were used to set the thickness of the gels. Each gel consisted of three portions: a 1 cm stacking gel, a 1.5 cm spacer gel, and a 3.0 cm separating gel.

All samples were dissolved in loading buffer and heated to 85 °C for 5 min prior to gel loading. The purified antibiotic was dissolved directly in loading buffer. BSA, porcine heart fumarase, carbonic anhydrase, β-lactoglobulin, α-lactalbumin,

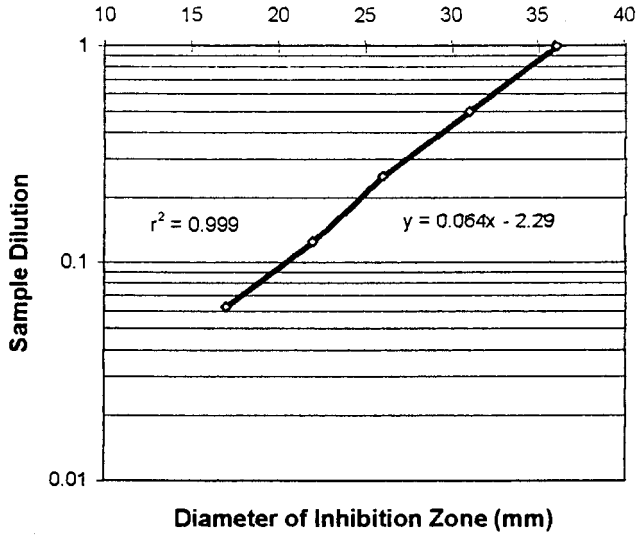

Figure 1. Relationship between peptide antibiotic concentration and resulting diameter of inhibition zone produced against *A. vitis*.

and bacitracin were used as molecular weight standards. All of the molecular weight standards were of electrophoresis grade except for bacitracin. Culture supernatant samples were lyophilized and dissolved in loading buffer prior to SDS−PAGE. Gels were run at 200 V until the tracking dye reached the bottom of the gel (~40 min).

Gels were visualized by staining with silver (Garfin, 1990). Antibiotic activity was specifically located on the gel using a direct activity assay (Bhunia et al., 1987).

Gel Filtration Chromatography. After review of the methods of Galvez et al. (1993), Muriana and Klaenhammer (1991), and Upreti and Hinsdill (1973), a method was devised for the gel filtration of the antibiotic. Approximately 1.5 mg of semipurified antibiotic was dissolved in 1 mL of 50 mM Tris-HCl, pH 7.5. One milliliter was applied to a 1.5 × 30 cm low-pressure chromatography column (Bio-Rad) filled with Bio-Gel P-10 (Bio-Rad) equilibrated in 50 mM Tris-HCl, pH 7.5. The antibiotic was eluted from the column using a flow rate of 0.5 mL/min at 4 °C. The eluent was monitored at 280 nm, and fractions were collected every 2.0 mL and assayed for activity using the agar-well diffusion assay. An Econo System low-pressure chromatography unit (Bio-Rad) was used to monitor the eluent, set the flow rate, and collect the fractions.

The void volume and column volume were determined using blue dextran 2000 (Pharmacia) and copper sulfate, respectively. Polymyxin B sulfate and streptomycin sulfate were used as molecular weight standards. Polymyxin B and streptomycin have molecular masses of 1300 and 1457 Da, respectively. All standards were made up as 1 mg/mL stocks and run under the same conditions as the sample.

RESULTS

Activity Assay. A sample of cell-free culture supernatant was used to demonstrate the relationship between the antibiotic concentration and the resulting zone of inhibition seen in the agar well-diffusion assay using *A. vitis*. Plotting the logarithm of sample dilution against the diameter of the zone of inhibition yields a linear response (Figure 1) as predicted by Betina (1983). Regression analysis of the data confirmed a linear relationship ($r^2 = 0.999$).

Microbial Spectrum. All of the Gram-negative bacteria tested were sensitive to the antibiotic, albeit at varying degrees (Table 1). The most sensitive Gram-negative bacterium was *A. vitis*, which demonstrated a zone of inhibition in excess of 30 mm in diameter when subjected to the agar diffusion assay. None of the Gram-positive organisms tested was markedly affected by the antibiotic. Each of them demonstrated a small zone of inhibition surrounding the well in the agar diffusion assay; however, this effect was considered to be negligible. Of the two fungi tested (*Penicillium expansum* and *Botrytis cinerea*), only *B. cinerea* showed susceptibility to the antibiotic.

Table 1. Sensitivity of 12 Microorganisms to the Antibiotic

microorganism	sensitivity[a]	microorganism	sensitivity[a]
E. coli	+	*A. vitis*	+++
S. typhimurium	+	*S. aureus*	−
E. amylovora	++	*L. monocytogenes*	−
P. corrugata	++	*B. cereus*	−
P. fluorescens	+	*P. expansum*	−
P. putida	+	*B. cinerea*	++

[a] (−) = zone of inhibition < 10 mm diameter; (+) = zone of inhibition = 10−20 mm diameter; (++) = zone of inhibition = 20−30 mm diameter; (+++) = zone of inhibition > 30 mm diameter.

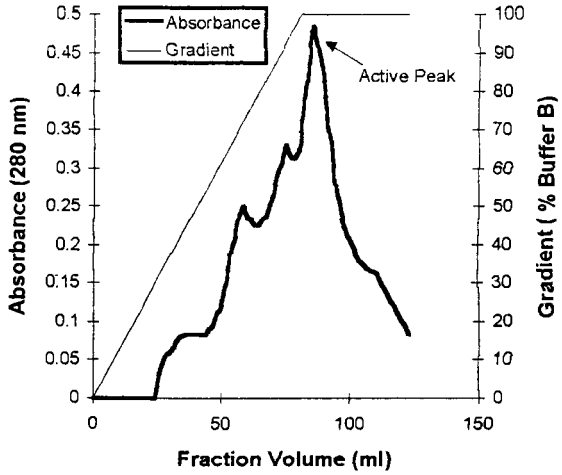

Figure 2. Elution pattern of the HCl precipitate from DEAE-Sephacel equilibrated in 50 mM Tris buffer, pH 7.5 (buffer A). The peptide was eluted from the column with a gradient of 0−100% buffer B (50 mM tris buffer, pH 7.5, containing 0.4 M NaCl). Flow rate = 0.5 mL/min.

Production and Purification of the Antibiotic. Purification of the biologically active component from the culture medium is summarized in Table 2. Initial concentration of the antibiotic was achieved by decreasing the pH of the culture supernatant to 2.0 with concentrated HCl. The rust-colored precipitate contained the antibiotic, and the pellet dissolved in 50 mM Tris-HCl, pH 7.5. The initial acid purification and centrifugation yielded 103% of the original activity and increased the specific activity 3.3-fold.

The antibiotic was retained by DEAE-Sephacel quite strongly but eluted from the matrix in 0.4 M NaCl. Ion exchange chromatography separated the crude antibiotic sample into four peaks (Figure 2). Antibiotic activity corresponds with the fourth and largest peak on the chromatogram. Only 23% of the initial activity was recovered by ion exchange chromatography, and the specific activity actually decreased relative to the that in previous purification step. However, the rust-colored contaminant was effectively and irreversibly bound to the ion exchange medium during this process. A complete loss of activity was observed when the antibiotic was left in the ion exchange eluent overnight (data not shown). For this reason the ion exchange protocol was carried out as quickly as possible at 4 °C.

Table 2. Purification of the Peptide Antibiotic from 50 mL of a 32-h Culture Supernatant of *B. subtilis*

sample	volume (mL)	total activity[a] (AU)	total absorbance ($A_{280\ nm}$)	specific activity (AU/A_{280})	activity recovered[b] (%)	fold purification
culture supernatant	50	3600	295	12		
HCl precipitate	5.0	3715	95	39	103	3.3
ion exchange peak	15	840	33	26	23	2.2
isobutanol extract	0.5	1208	13	94	34	7.8
HPLC peak	0.5	660	7	97	18	8.1

[a] Activity is expressed as activity units (AU) against *A. vitis* and was determined using the agar diffusion assay. [b] Percentage of initial activity.

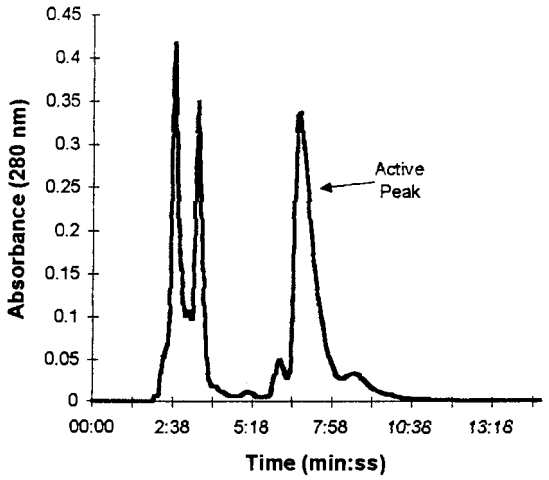

Figure 3. Purification of the peptide antibiotic by reversed-phase HPLC on a Pharmacia C_{18} column. The sample applied to the column was the butanol extract of the ion exchange peak. Eluent was acetonitrile/1% acetic acid (68:32). Flow rate = 1 mL/min.

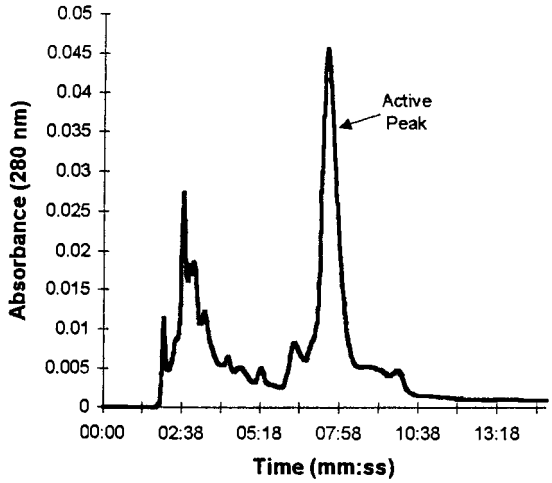

Figure 4. Repurification of the peptide antibiotic by HPLC. The active peak from the initial HPLC chromatogram (Figure 3) was collected, concentrated, and repurified on a Pharmacia C_{18} reversed-phase column. Eluent was acetonitrile/1% acetic acid (68:32). Flow rate = 1 mL/min.

Isobutanol extraction of the pooled activity from ion exchange purification desalts and concentrates the antibiotic. Upon drying of the isobutanol extract, the antibiotic preparation is an amorphous pale yellow powder. Isobutanol extraction affords a 34% recovery of the initial activity and increases the initial specific activity nearly 8-fold (Table 2). The butanol extract served as the working form of the antibiotic for all other analyses excluding the amino acid analyses.

HPLC purification of the antibiotic provided a highly purified sample for amino acid analysis. Figure 3 details a typical HPLC chromatogram of the butanol extract of the antibiotic. A substantial amount of ultraviolet absorbing material elutes in the early stages of the purification. When the active peak from the HPLC purification is collected and repurified according to the same process, the resulting chromatogram (Figure 4) is remarkably similar to the original one (Figure 3). HPLC purification results in an 18% recovery of the initial antibiotic activity and increases the initial specific activity 8.1-fold over that of the original sample.

Chemical Characteristics. The absorbance spectrum for the antibiotic is measured in acetonitrile/1% acetic acid (68:32) between 200 and 600 nm. The antibiotic shows absorbance maxima at 235, 278, and 285 nm (Figure 5), and there is no appreciable absorbance above 300 nm.

The FTIR spectrum of the purified antibiotic is shown in Figure 6. Characteristic absorption valleys at 1540, 1650, and 3300 cm^{-1} indicate that the antibiotic contains peptide bonds. A lactone ring is suggested by the absorption at 1740 cm^{-1}, and valleys that result from C−H stretching (2950, 2850, 1460, 1400 cm^{-1}) indicate the presence of an aliphatic chain.

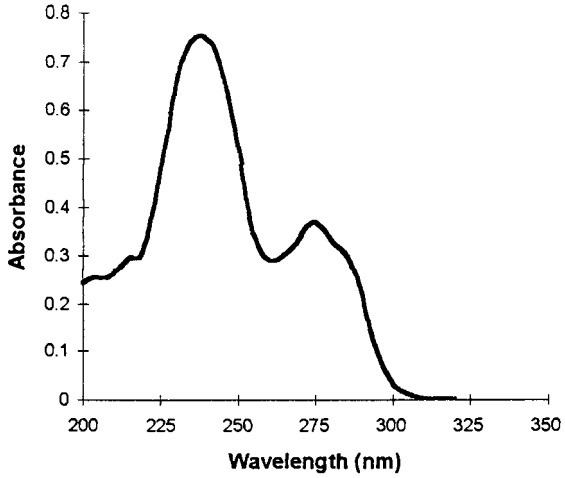

Figure 5. Ultraviolet spectrum of the peptide antibiotic in acetonitrile/1% acetic acid (68:32).

The amino acid composition is detailed in the insert to Figure 6. Sixteen peaks were identified by the amino acid analyzer; however, only eight were above the threshold value. The total amino acid composition was determined to be aspartic acid, glutamic acid, serine, glycine, alanine, proline, valine, and leucine in a ratio of 2:3:1:1:1:1:1:4.

Gel Electrophoresis. SDS−PAGE (Figure 7) confirmed the purity of the antibiotic preparation. The antibiotic (lane 3) is considerably smaller than any of the molecular weight standards run in lane 1, the smallest of which was α-lactalbumin at a molecular mass of 14 200 Da. Rather, it migrates at a rate similar to that of bacitracin (lane 4), which is a cyclic peptide antibiotic with a molecular mass of 1486 Da.

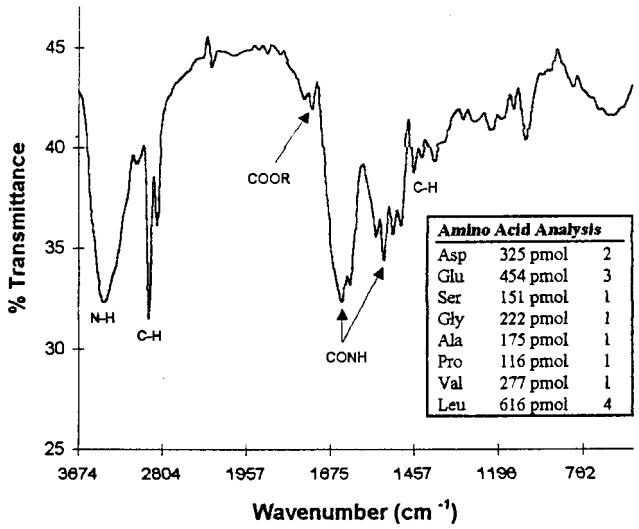

Figure 6. Fourier transform IR spectrum (KBr pellet) and amino acid composition of the peptide antibiotic.

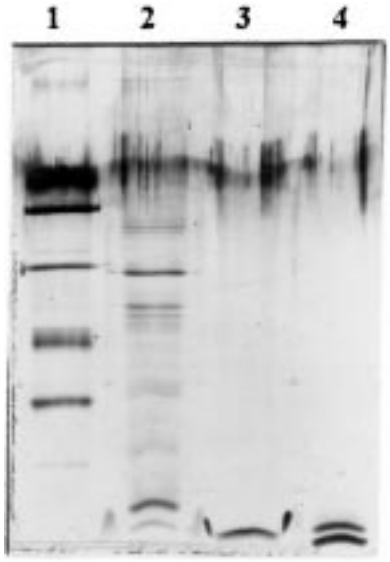

Figure 7. SDS−PAGE of peptide antibiotic: (lane 1) molecular mass standards (BSA = 66 kDa, porcine heart fumarase = 48.5 kDa, carbonic anhydrase = 29 kDa, β-lactoglobulin = 18.4 kDa, α-lactalbumin = 14.2 kDa); (lane 2) culture supernatant; (lane 3) semipurified antibiotic; (lane 4) bacitracin (1.5 kDa).

The antibiotic (lane 3) is free of any contaminating material as evidenced by the lack of any other bands. As a comparison, the culture supernatant (lane 2) produces many bands of stained contaminating material. Evidently, all of this contaminating material is removed by the purification protocol.

A direct activity assay (Figure 8) confirms that the lone band in lane 3 (Figure 7) has antibiotic activity. In other words, the zone of inhibition seen in the direct activity assay corresponded to the location of the band in lane 3. Clearly, the antibiotic retained its activity even after being subjected to the conditions of SDS−PAGE.

Gel Filtration. Gel filtration of the semipurified antibiotic was accomplished using Bio-Gel P-10 as the column matrix (Figure 9). Early attempts at gel filtration using a Sephadex matrix indicated that the peptide had affinity for the Sephadex (data not shown) and antibiotic activity eluted after the total column volume.

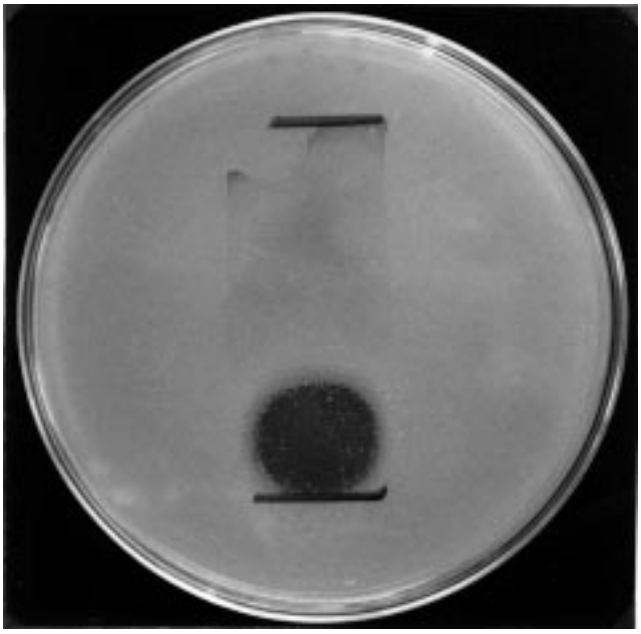

Figure 8. Direct detection of peptide antibiotic on SDS−polyacrylamide gel. A sample of culture supernatant was run in an outside lane of the gel. The lane was cut out, fixed, washed, and overlaid with soft agar seeded with *A. vitis*. The plate was incubated overnight.

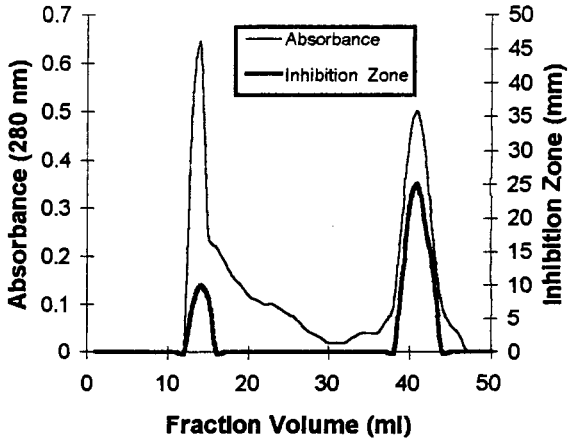

Figure 9. Elution pattern of peptide antibiotic from a Bio-Gel P-10 column equilibrated in 50 mM Tris buffer, pH 7.5. Fractions were collected and measured for activity (zone of inhibition). Flow rate = 0.5 mL/min. Void volume = 14 mL (blue dextran). Column volume = 45 mL (copper sulfate). Molecular mass standards were streptomycin (1457 Da) and polymyxin B (1300 Da) eluted at 43 and 44 mL, respectively.

The antibiotic elutes from the column at two distinct peaks. On the basis of the elution volumes of streptomycin and polymyxin (43 and 44 mL, respectively), the fastest migrating form of the antibiotic (elution volume = 42 mL) has an apparent molecular mass of ~1500 Da. However, antibiotic activity also eluted from the column at the void volume (14 mL), indicating that the antibiotic was also present in a form with an apparent molecular mass in excess of 20 000 Da.

DISCUSSION

This paper describes the purification and partial chemical characterization of an antimicrobial compound produced by a bacterium that has been identified as an isolate of *B. subtilis* (Sholberg et al., 1995). This particular organism was chosen for study for two

reasons: it was isolated as an endophyte from the internal tissue of apple fruit, and it demonstrated inhibitory properties in a biocontrol assay against blue and gray mold decay in apples (Sholberg et al., 1995).

Table 2 outlines the effectiveness of the purification protocol. On the basis of these results, acid precipitation of the culture supernatant and isobutanol extraction of the ion exchange peak actually increased antibiotic activity relative to the previous step. Recovered activity is not necessarily a good indication of antibiotic recovery because activity may be affected by buffer conditions and puriy, which are different at each stage of the purification. An increase in the recovery of activity from one purification step to the next is not unusual. Galvez et al. (1986) noted a 118% recovery of activity in the peptide antibiotic AS-48 after gel filtration, whereas Smidt and Vidaver (1986) reported a 115% recovery in the activity of syringacin W-1 upon concentration of the ultrafiltration retentate.

Ion exchange purification of the acid precipitate seems to be detrimental to the activity and stability of the antibiotic. At the expense of decreasing the activity of the antibiotic, the ion exchange protocol was very effective at eliminating colored material contaminating the sample, and it separated the antibiotic from three other peaks seen in the chromatogram.

HPLC purification of the antibiotic is quite effective (Figure 3). There is a fair amount of contaminant in the antibiotic preparation that eluted in the early stages of the HPLC chromatogram (retention time of ~3 min). When the active peak from the initial HPLC run was collected and repurified according to the same method, the resulting chromatogram (Figure 4) was remarkably similar to the initial one. The material eluting early from the HPLC column may be a derivative or a degradation product of the antibiotic.

The ultraviolet spectrum of the antibiotic (Figure 5) suggests that it is a peptide; however, the absorbance pattern is not characteristic of most peptide antibiotics, which produce absorbance maxima at 210−230 nm. Rather, the peptide antibiotic gives absorbance maxima that are very similar to that of the peptide antibiotic LI-FO3 produced by *Bacillus polymyxa* (Kurusu and Ohba, 1987). According to Kurusu and Ohba (1987), this absorbance pattern indicates the presence of a tyrosine residue. Similar ultraviolet spectra were demonstrated with enduracidin (Asai et al., 1968) and janiemycin (Meyers et al., 1970). Neither of these peptides contains tyrosine in its structure; however, both contain α-amino-4-hydroxyphenylacetic acid, which is very similar to tyrosine.

The FTIR spectrum of the peptide antibiotic (Figure 6) offers valuable information about the peptide antibiotic. Analysis of the spectrum offers the first concrete evidence that the antibiotic contained a peptide in its structure; however, the spectrum also suggests the presence of an aliphatic chain and a lactone bond. On the basis of these findings, the peptide antibiotic in question appears to be very similar to a class of peptide antibiotics called peptolides. Peptolides are peptide antibiotics that contain a lactone bond in their structure and as a result are cyclic (Berdy, 1974). Furthermore, the peptide in question appears to belong to a subclass of peptolides that contain fatty acids. The FTIR spectrum of the peptide in question is nearly identical to that of several other such peptolides. Examples include arthrofactin (Morikawa et al., 1993), surfactin (Arima et al., 1968), and JF-2 lipopeptide (Lin et al., 1994). All of these compounds are peptides, contain a fatty acid moiety, and have a lactone ring in their structure. As well, they all demonstrated strong surface active properties and for this reason are sometimes classified as biosurfactants.

Amino acid analysis (Figure 6) identified 16 peaks in the antibiotic sample; however, only 8 of them were above the threshold value. On the basis of these results, the antibiotic contains aspartic acid, glutamic acid, serine, glycine, alanine, proline, valine, and leucine (2:3:1:1:1:1:1:4). Because acid hydrolysis converts the side chains of asparagine and glutamine to aspartate and glutamate (Rawn, 1989), the molar ratios of aspartic acid and glutamic acid refer to the combined totals of either aspartic acid/asparagine or glutamic acid/glutamine, respectively. On the basis of the acidic properties of the antibiotic (precipitates at pH 2.0) and the lack of other charged amino acids in the structure, it is likely that both of these amino acid residues were present predominantly in the acidic form. The presence of tyrosine is suggested by the ultraviolet spectrum of the peptide, but this is not confirmed by the amino acid analysis. The ultraviolet absorbance pattern could be the result of some unusual aromatic amino acid that was produced as an artifact during the amino acid analysis. It is also possible that the quantity of tyrosine was underestimated by the analysis. Approximately one-third of the amino acids in the peptide are charged, whereas the remaining two-thirds are hydrophobic. This type of charged/hydrophobic structure can impart some interesting properties to the peptide antibiotic, mainly surface active properties.

From amino acid analysis, the estimated molecular mass of the peptide moiety of the antibiotic is 1320 Da or a multiple thereof. The unit mass of 1320 Da is consistent with the migration of the antibiotic during SDS−PAGE.

Gel filtration of the peptide antibiotic (Figure 9) indicates that the peptide antibiotic eluted from the Bio-Gel column in two different forms. It eluted as a monomer (MW ~ 1500 Da) and as an aggregate (MW > 20 000 Da). Because the antibiotic sample applied to the gel filtration column was essentially free of other proteinaceous material as indicated by SDS−PAGE, the peptide antibiotic must have aggregated, perhaps to form some type of micelle structure. The formation of micelles by peptide antibiotics is a common occurrence and is often associated with peptides that demonstrate surface active properties such as surfactin (Arima et al., 1968), arthrofactin (Morikawa et al., 1993), and biosurfactants produced by *Bacillus licheniformis* (Lin et al., 1994; Jenny et al., 1991). Each of these compounds is a lipopeptide, demonstrates surface active properties, and forms high molecular weight aggregates. Mulligan and Gibbs (1990) took advantage of this last property in a one-step method to purify and concentrate surfactin from the culture supernatant by ultrafiltration. The ability of surfactant molecules to form high molecular aggregates allows them to be retained by relatively high molecular weight cutoff membranes.

Surface active properties (Jain et al., 1991) were evident for the peptide antibiotic in this study (data not shown). When samples of purified peptide antibiotic dissolved in distilled water were dropped onto a hydrophobic surface (Parafilm), droplets did not form; rather, the water formed a sheet on the surface of the Parafilm.

When pure distilled water was dropped onto the same surface, droplets formed that could not be disrupted. This result is indicative of a significant decrease in the surface tension of the distilled water containing the peptide antibiotic.

The antibiotic demonstrates interesting antimicrobial properties. It appears to have a broad spectrum of activity against Gram-negative organisms, shows little activity against Gram-positive organisms, and is active against one of the two fungi assayed. More Gram-negative bacteria and fungi need to be assayed to fully characterize the antimicrobial spectrum of the antibiotic. Studies on the effect of the antibiotic on *A. vitis* cells suggest a bactericidal mode of action rather than bacteriostatic effect; however, cell death does not appear to be associated with cell lysis.

In summary, the antibiotic isolated and partially characterized in this study is an acidic peptide containing eight different amino acids. The molecular mass of the compound is 1500 Da but aggregates in excess of 20 000 Da can be formed. The peptide antibiotic also contains an acyl chain and has a lactone bond in its structure. Empirical evidence suggested that the lipopeptide antibiotic demonstrated surface active properties. On the basis of the results of this study, the lipopeptide antibiotic in question appears to be similar to a number of peptide antibiotics often termed "biosurfactants" and classified according to the guidelines in Berdy (1974) as fatty acid-containing peptolides. The antibiotic demonstrated a broad spectrum of activity against Gram-negative bacteria, shows little activity against Gram-positive organisms, and is active against one of two fungi assayed.

LITERATURE CITED

Arima, K.; Kakinuma, A.; Tamura, G. Surfactin, a crystalline peptidelipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation. *Biochem. Biophys. Res. Commun.* **1968**, *31*, 88−494.

Asai, M.; Moroi, M.; Sugita, N.; Kawashima, H.; Mizuno, K.; Miyake, A. Enduracidin, a new antibiotic. Isolation and characterization. *J. Antibiot.* **1968**, *21*, 138−146.

Berdy, J. Recent developments of antibiotic research and classification of antibiotics according to chemical structure. *Adv. Appl. Microbiol.* **1974**, *18*, 309−406.

Besson, F.; Michel, G. Mycosubtilins B and C: minor antibiotics from mycosubtilin producing *Bacillus subtilis*. *Microbios* **1990**, *62*, 93−99.

Betina, V., Ed. *The Chemistry and Biology of Antibiotics*; Elsevier Scientific Publishing: New York, 1983.

Bhunia, A. K.; Johnson, M. C.; Ray, B. Direct detection of an antimicrobial peptide of *Pediococcus acidilactici* in sodium dodecyl polyacrylamide gel electrophoresis. *J. Ind. Microbiol.* **1987**, *2*, 319−322.

Brock, T. D., Madigan, M. T., Eds. *Biology of Microorganisms*, 6th ed.; Prentice Hall: Englewood Cliffs, NJ, 1991.

Delcambe, L.; Devignat, R. L'Iturine, nouvel antibiotique d'origine congolaise. *Acad. Sci. Coloniales* **1957**, *6*, 1−77 (in Appl. Microbiol. Biotechnol. **1993**, *39*, 438−442.)

Fiechter, A. Biosurfactants: moving towards industrial application. *Trends Food Sci. Technol.* **1992**, *3*, 286−293.

Galvez, A.; Maqueda, M.; Valdivia, E.; Quesada, A.; Montoya, E. Characterization and partial purification of a broad spectrum antibiotic AS-48 produced by *Streptococcus faecalis*. *Can. J. Microbiol.* **1986**, *32*, 765−771.

Galvez, A.; Maqueda, M.; Martinez-Bueno, M.; Lebbadi, M.; Valdivia, E. Isolation and physico-chemical characterization of an antifungal and antibacterial peptide produced by *Bacillus licheniformis* A12. *Appl. Microbiol. Biotechnol.* **1993**, *39*, 438−442.

Garfin, D. E. One-dimensional gel electrophoresis. In *Guide to Protein Purification, Methods in Enzymology*; Deutscher, M. P., Ed.; Academic Press: New York, 1990; Vol. 182, pp 425−440.

Hozono, K.; Suzuki, H. Acylpeptides, the inhibitors of cyclic adenosine 3′,5′-monophosphate phosphodiesterase. *J. Antibiot.* **1983**, *36*, 667.

Jain, D. K.; Thompson, D. L. C.; Lee, H.; Trevors, J. T. A drop-collapsing test for screening surfactant producing microorganisms. *J. Microbiol. Methods* **1991**, *13*, 271−279.

Jenny, K.; Kappeli, O.; Fiechter, A. Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Appl. Microbiol. Biotechnol.* **1991**, *36*, 5−13.

Katz, E.; Demain, A. L. The peptide antibiotics of *Bacillus*: chemistry, biogenesis, and possible functions. *Bacteriol. Rev.* **1977**, *41*, 449−474.

Kurusu, K.; Ohba, K. New peptide antibiotics LI-FO3, FO4, FO5, FO7, and FO8, produced by *Bacillus polymyxa*. I. Isolation and characterization. *J. Antibiot.* **1987**, *40*, 1506−1514.

Lin, S. C.; Carswell, K. S.; Sharma, M. M.; Georgiou, G. Continuous production of the lipopetide biosurfactant of *Bacillus licheniformis* JF-2. *Appl. Microbiol. Biotechnol.* **1994**, *41*, 281−285.

McKeen, C. D.; Reilly, C. C.; Pusey, P. L. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. *Phytopathology* **1986**, *76*, 136−139.

Meyers, E.; Weisenborn, F. L.; Pansy, F. E.; Slusarchyk, D. S.; von Saltza, M. H.; Rathnum, M. L.; Parker, W. L. Janiemycin, a new peptide antibiotic. *J. Antibiot.* **1970**, *23*, 503−507.

Morikawa, M.; Daido, H.; Takao, T.; Murata, S.; Shimonishi, Y.; Imanaka, T. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. *J. Bacteriol.* **1993**, *175*, 6459−6466.

Mulligan, C. N.; Gibbs, B. F. Recovery of biosurfactants by ultrafiltration. *J. Chem. Technol. Biotechnol.* **1990**, *47*, 23−29.

Muriana, P. M.; Klaenhammer, T. R. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. *Appl. Environ. Microbiol.* **1991**, *57*, 114.

Peypoux, F.; Besson, F.; Michel, G.; Delcambe, L. Structure of bacillomycin D, a new antibiotic of the iturin group. *Eur. J. Biochem.* **1981**, *118*, 323−327.

Rawn, D. J., Ed. *Proteins, Energy, and Metabolism*; Neil Patterson Publishers: Burlington, NC, 1989.

Schagger, H.; von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *26*, 368−379.

Sholberg, P. L.; Marchi, A.; Bechard, J. Biocontrol of postharvest diseases of apple using *Bacillus* spp. isolated from stored apples. *Can. J. Microbiol.* **1995**, *41*, 247−252.

Smidt, M. L.; Vidaver, A. K. Isolation and characterization of syringacin W-1, a bacteriocin produced by *Pseudomonas syringae* pv. *syringae*. *Can. J. Microbiol.* **1986**, *32*, 231−236.

Tagg, J. R.; McGiven, A. R. Assay system for bacteriocins. *Appl. Microbiol.* **1971**, *21*, 943.

Upreti, G. C.; Hinsdill, R. D. Isolation and characterization of a bacteriocin from a homofermentative *Lactobacillus*. *Antimicrob. Agents Chemother.* **1973**, *4*, 487.

von Döhren, H. Peptides. In *Genetics and Biochemistry of Antibiotic Production*; Vining, L. C., Stuttard, C., Eds.; Butterworth-Heinemann: Newton, MA, 1995; pp 129−171.

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