Purification and characterization of Pseudomycin, a phytotoxin and antimycotic produced by Pseudomonas syringae
by Leslie Ann Harrison

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Plant Pathology
Montana State University


Abstract:
Many microorganisms produce secondary metabolites which affect other organisms sharing their ecological niche. Such may be the case when a plant pathogen invades its host. Pseudomonas syrinqae. a plant pathogenic bacterium, produces a number of such metabolites one of which has not been previously purified and identified.

This thesis describes the isolation and characterization of a small peptide from Pseudomonas svrinqae called pseudomycin. The peptide was purified using acetone precipitation, standard column chromatography, and high preformance liquid chromatography. Final characterization was by means of fast atom bombardment (FAB) mass spectroscopy and amino acid analysis.

Once purified, interest lies in the biological activity of the compound. Pseudomycin was tested on a number of monocots and dicots and found to be selectively phytotoxic. In addition this peptide formed zones of inhibition when oversprayed with suspensions of fungal mycelia or bacteria, demonstrating antimicrobial activity against both plant and human pathogens.

Microbial metabolites may or may not play a role in plant disease. By studying bacterial metabolites we may gain a greater understanding of host/pathogen interactions in plant disease and therefore evaluate effective treatment. Applications may also include use as selective natural product herbicides or fungicides for the treatment of plant or human disease.

# PURIFICATION AND CHARACTERIZATION OF PSEUDOMYCIN, A PHYTOTOXIN AND ANTIMYCOTIC PRODUCED BY *PSEUDOMONAS SYRINGAE*

by

Leslie Ann Harrison

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## ABSTRACT

Many microorganisms produce secondary metabolites which affect other organisms sharing their ecological niche. Such may be the case when a plant pathogen invades its host. Pseudomonas syringae, a plant pathogenic bacterium, produces a number of such metabolites one of which has not been previously purified and identified.

This thesis describes the isolation and characterization of a small peptide from Pseudomonas syringae called pseudomycin. The peptide was purified using acetone precipitation, standard column chromatography, and high preformance liquid chromatography. Final characterization was by means of fast atom bombardment (FAB) mass spectroscopy and amino acid analysis.

Once purified, interest lies in the biological activity of the compound. Pseudomycin was tested on a number of monocots and dicots and found to be selectively phytotoxic. In addition this peptide formed zones of inhibition when oversprayed with suspensions of fungal mycelia or bacteria, demonstrating antimicrobial activity against both plant and human pathogens.

Microbial metabolites may or may not play a role in plant disease. By studying bacterial metabolites we may gain a greater understanding of host/pathogen interactions in plant disease and therefore evaluate effective treatment. Applications may also include use as selective natural product herbicides or fungicides for the treatment of plant or human disease.

# CHAPTER I

## INTRODUCTION

The pseudomonad group of bacteria are found ubiquitously as free living saprophytes in soils, water, and in association with both plants and animals. These organisms play roles which can be at opposite ends of the biological spectrum. Some pseudomonad strains actively mineralize organic material, a great benefit to the economy of nature (25). At the same time many are causative agents of disease in man, animals, and plants.

Numerous diseases with a wide range of symptoms may be caused by phytopathogenic pseudomonads. These include necrotic lesions and spots on fruit, stems, and leaves; hyperplasias (galls,scabs); tissue maceration (rots); cankers; blights; and vascular infections (wilts). Pseudomonad incited plant diseases can be found worldwide and affect most major groups of plants (25).

Pseudomonas syringae is principally known as a group of unspecialized foliar pathogens which survive in association with the host plant and propagative material of the host plant. Bacterial blight caused by P. syringae van Hall is a foliar disease of wheat (Triticum aestivum L.), in the northern United States (43). This disease affects spring

and winter wheat in Minnesota, South Dakota, and Montana (32,34). Yield losses from bacterial leaf blight are incompletely assessed, but foliage destruction may exceed 50% under ideal environmental conditions (43). The symptoms include grey green necrosis and bleaching of leaves, glumes, sheaths, and culms. This disease is more often found in wheat, but is occasionally found in barley (16).

P. syringae in stone and pome fruits exists in lesions, cankers, or tumors. One of the more serious canker diseases, particularly in California, is caused by P. syringae. This bacterium is most active in the fall and winter months, producing cankers which originate primarily in nodal areas. After infection, the bacterial cells multiply and move quickly through cortex tissues producing cankers which appear as depressed, water soaked areas, with a brown color and sour smell (36).

There are at least two features which enhance the pathogenicity of P. syringae (36). First, in culture and in infected plant tissue it may produce a toxin that destroys host cell membranes and thus contributes to symptom development (5,7). The site of action of one of these toxins, syringomycin, has been investigated. Bidwai, et al.(2), demonstrated preferential stimulation of vanadate-sensitive ATPase activity of the plasma membrane of red beet storage tissue. The second feature is the capability of some strains to act as nuclei for formation of ice crystals.

At temperatures just below 0° C, most plants that grow in temperate zones escape significant frost damage because water in their tissues remains in liquid form, supercooled. If ice nucleating bacteria are present, ice crystals form and disrupt plant tissue, resulting in typical disease symptoms. P. syringae in combination with freezing conditions causes severe damage to plants that would not be harmed by either agent alone (36).

Symptoms produced as a result of bacterial toxins have been documented since the early portion of the twentieth century. Identification of the chemical nature of such toxins, however, has been very limited. Prior to 1970 no correct toxin structure had been published (21). One of the first investigations was that by Braun and Woolley of the wildfire toxin (tabtoxin) and its involvement in the wildfire disease of tobacco (21). In recent years chemical structures of toxins from a number of bacterial pathogens have been elucidated.

There has been a number of definitive studies concerning toxin production as a part of the disease process in Pseudomonas infections. For example, halo blight of beans, caused by P. phaseolicola, is mediated by a toxin (12). Several researchers characterized phaseolotoxin from P. phaseolicola, as a tripeptide composed of ornithine, alanine, and homoarginine with a phosphosulfamyl group attached to the ornithine (20,38,39). A number of other

toxins are elaborated by members of the P. syringae group; including tabtoxin (28,29,37), coronatine (19,23), syringomycin, syringotoxin (10), tagetitoxin (18,22), and several that are still uncharacterized. The strains that produce these toxins and the structural characteristics are summarized in a review by Mitchell (21).

Those toxins that are of particular interest in relation to this study are syringomycin and syringotoxin. These two compounds are peptidyl, broad spectrum antibiotics and phytoxins isolated from ecotypic strains of P. syringae. Preparations of syringomycin yielded amino acids tentatively identified as serine, phenylalanine, an unidentified basic amino acid and arginine in a 2:1:2:1 molar ratio (10). The original structure was confirmed and the unidentified amino acid was identified as 2,4-diaminobutyric acid in studies reported in a recent paper by Ballio et al. (1). Syringotoxin, isolated from a strain of P. syringae from a citrus host, contained substances tentatively identified as threonine, serine, glycine, ornithine, and the again originally unidentified amino acid, 2,4-diaminobutyric acid (21).

Dr. Bruce Hemming, a former researcher in our laboratory, demonstrated the production of a toxin by an isolate of P. syringae, obtained from barley (Hordeum vulgare), which showed many similarities to syringomycin and syringotoxin. The partially purified toxin was found to be

ninhydrin positive, have a small molecular weight, and demonstrated antimycotic activity (11).

Thus, the first goal of this research project was to develop a method of purification for this toxin. Previous attempts at thin layer chromatography and high performance liquid chromatography separation had failed. Initial toxin preparations seemed to be fairly unstable which confounded the interpretation of data. The method of choice for purification must separate the toxin or unknown toxin from all other components without drastically altering its bioactivity.

The second goal was to biologically characterize this toxin. This toxin and several previously described toxins, showed antimycotic activity. It is necessary to know which of the plant pathogenic fungi are sensitive to the toxin to consider application. The same is true for human fungal pathogens.

# CHAPTER II

## PURIFICATION AND CHEMICAL CHARACTERIZATION OF PSEUDOMYCIN

### Introduction

Before any biological activity can be studied in depth a certain line of cause and effect must be established. Is the effect due to the compound of interest or due to a small contaminant carried along in the procedure? If an unknown can be isolated to purity, then identified, this cause and effect relationship can be established.

Purification of the toxin, tentatively called pseudomycin, has been a challenge due to loss of biological activity at various stages of purification. Conditions such as pH, heat stability, and solvent solubilities must be considered. In addition, there are problems such as ionic exchange or nonspecific irreversible binding to glass, silica, or even column packing material.

During the experimental stages of purification, a variety of methods were attempted for the separation of pseudomycin. For example, a common step in separation science before high performance liquid chromatography, is preparative thin layer chromatography. This method (Appendix), was attempted with some success, but proved

inefficient when compared to later techniques. The main problem seemed to be lack of elution of the sample from the silica. Successful elution was accomplished eventually by using 3N hydrochloric acid or 3N trifluoroacetic acid as the eluant. Surprisingly, this did not appreciably decrease the activity of the compound.

Alternate methods for toxin purification were used with varying results. Some of these techniques may be useful in future research. For this reason several are briefly presented in the Appendix.

Presented in this chapter is a purification scheme which is fairly uncomplicated and results in several milligrams of purified toxin per liter of culture medium extracted. Following the description of the purification are the chemical analyses that led to the identification of a small peptidyl phytotoxin.

## Materials and Methods

### Bacterial Strains

Pseudomonas syringae MSU 174, isolated from a Montana barley field, was obtained from Dr. David Sands, Montana State University. In an attempt to increase toxin production, MSU 174 was mutagenized by transposon mutagenesis using TN 905 (13). A "super producer" was isolated and was called strain 206. Strain 206 was used by Dr. Rudy Scheffer to test the biocontrol aspects of

P.syringae against Dutch Elm Disease. One year after the initiation of the experiment, samples were taken from the tree branches for the reisolation of strain 206. Strain 206 was reisolated and positively identified by Southern blot techniques. The strain now produced a larger zone of inhibition with the bioassay and was labeled P. syringae MSU 16H. This is the strain that was used in the purification of pseudomycin.

P. syringae MSU 16H was stored in 15% glycerol at -70° C. Transfers were made every two weeks to Kings B media (Appendix) and grown at room temperature.

Butanol Extraction

P. syringae 16H was grown in shake culture (180 rpm), for six days at 23° C in Dyes media supplemented with $FeCl_3$, ornithine, and histidine (Appendix). Cultures were centrifuged at 5000 X g for 10 min. to pellet the cells. The supernatant liquid was flash evaporated to 200 ml at 38° C, then extracted three times with an equal volume of 1-butanol. The butanol fraction was extracted with water, then the butanol phase was flash evaporated to dryness at 38° C. The dried concentrate was resuspended in methanol for transfer, then dried under nitrogen for storage at -20° C (Appendix, Figure 8).

Acetone precipitation

Cells were grown in PDB as a still culture at 23° C for

6 days. Cultures were mixed 1:1 (v/v) with acetone then centrifuged at 5000 X g for 10 minutes. The liquid supernatant was flash evaporated to 200 ml then brought to a final concentration of 60% acetone. This mixture was allowed to precipitate overnight at 4° C with gentle stirring. The precipitate was removed by centrifugation at 5000 X g for 10 minutes. The liquid supernatant was flash evaporated to dryness then resuspended in one liter of 0.1% TFA (Appendix, Figure 9).

Amberlite column

Amberlite XAD-2 (mesh size 20-60) was washed with 0.1% TFA, then packed in a 1.6 X 40 cm column. The column was equilibrated with 0.1% TFA. The sample that was resuspended in one liter 0.1% TFA (butanol or acetone procedure), was loaded onto the column at 1 ml/min. with a Waters M25 solvent delivery system. Pseudomycin was eluted with a nonlinear gradient of 0 to 100% 1-propanol with 0.1% TFA according to Bidwai, et al. (2) (Appendix, Figure 10). The gradient former was a Kratos Spectroflow 430. Fractions were collected with a Gilson microfractionator and tested for bioactivity. Active fractions were pooled, dried with the rotary evaporator, and resuspended in 50% 1-propanol containing 0.1% TFA. Final drying of small quantities, before storage at -20° C, was accomplished by placing the sample under a flow of nitrogen gas.

Geotrichum candidum bioassay

The sample (10 μg) was applied to a PDA plate (Appendix) and allowed to dry. The plate was oversprayed with a sterile water suspension of Geotrichum candidum, sealed with parafilm, and incubated overnight at room temperature. Zones of complete inhibition were noted.

Geotrichum candidum was kindly provided by Dr. Don Mathre, Montana State University.

HPLC Purification

Active fractions from the Amberlite column were filtered through a 0.2 micron filter (Gelman Sciences) then subjected to reverse phase high performance liquid chromatography (RP-HPLC) on a 4.6 X 100 mm C8 column (Amicon MC-250) with a 1-propanol, nonlinear gradient (0 to 30% 1-propanol, 0.1% TFA, Waters #2 gradient over 35 min.). The flow rate of the mobile phase was 1 ml/min. Each peak was collected, evaporated, and resuspended in 50% 1-propanol with 0.1% TFA before being bioassayed. Single active peaks were combined then reinjected for further purification.

Collections from a single peak were again subjected to RP-HPLC utilizing a second solvent system. The sample was eluted with a linear, acetonitrile, 0.1% TFA gradient (Waters #6, 0 to 80% acetonitrile, 0.1% TFA) over a 20 minute period.

Purification of larger quantities of pseudomycin was done on a 10 X 250 mm Amicon MC-250 C8 preparative column with a flow rate of 2 ml/min.

With both columns, a Waters system was used including a model 440 absorbance detector monitoring at a wavelength of 254, and a model 660 solvent programmer. Two solvent delivery systems were required, a model M45 and a model A6000.

Collections from each peak to be used for biological testing were flash evaporated and resuspended in 50% 1-propanol containing 0.1% TFA. Samples for mass spectrometry were flash evaporated then concentrated, but not dried, under nitrogen. Samples for amino acid analysis were collected from the HPLC directly into polypropylene cryo-vials, and assayed directly without further concentration.

Syringomycin

Purified syringomycin was kindly provided by Dr. John Takemoto at Utah State University.

FAB Mass Spectrometry

Fast atom bombardment (FAB) mass spectrometry was performed by Joe Sears, department of chemistry, Montana State University. Analysis was with a VG MassLab Trio2 automated mass spectrometer connected to a Hewlett Packard model 5890 gas chromatograph. The column was a 30 meter P-5 microcolumn. The sample was run in both a glycerol and

thioglycerol matrix.

Nuclear Magnetic Resonance

The proton nuclear magnetic resonance (NMR) spectrum was recorded on a 500 MHz Bruker spectrometer. Chemical shifts were recorded in ppm units relative to trimethylsilane (0 ppm) with $D_2O$. NMR spectrometry analysis was kindly provided by Dr. Ed Dratz, department of chemistry, Montana State University.

Absorbance Characteristics

Pseudomycin, dissolved in 0.1% TFA, was scanned on a Beckman DU-50 Spectrophotometer. The solution (100 µl) was weighed on a Cahn model G electrobalance to obtain a value for the calculation of the molar concentration. The molar extinction coefficient was calculated using the maximum absorbance and the molar concentration. The scan and weighing were repeated three times and the average result was recorded.

Thin Layer Chromatography

The RP-HPLC sample of pseudomycin (3 µg) was spotted onto a silica gel 60 F254 thin layer plate (E. Merck Science). The same amount of syringomycin was spotted adjacent to the pseudomycin for a comparative standard. TLC plates were run in three separate solvent systems; 1) 1-butanol:pyridine:acetic acid:water, 2) 1-butanol:2-

picoline:acetic acid:water, and 3) 1-butanol:2,6-lutidine:acetic acid:water. The ratio was the same for each system, 15:10:3:12, (v/v). TLC plates were sprayed with ninhydrin reagent; 0.5% ninhydrin in 95% ethanol (v/v). The compounds appeared as light purple spots.

Amino Acid Analysis

Samples for amino acid analysis and sequencing were sent to Dr. David Teplow at the California Institute of Technology. All of the instrumentation for both amino acid analysis and sequencing was by Applied Biosystems Inc. For the amino acid analysis, a model 420 derivatizer coupled to a model 130 HPLC system was utilized. Data was acquired and analyzed by a model 920 software system. Amino acid sequencing was done with a model 477 sequencer with on line PTH amino polythiohydantoin (PTH) amino acid analysis. Peak quantitation was performed by software supplied with the model 477 sequencer. Peak identification was done manually by comparison to standard chromatograms.

Heat Stability

A 1-butanol crude extract (75 $\mu$l) of pseudomycin, at a concentration of 33 $\mu$g/$\mu$l, was placed into each of eight 1.5 ml Eppendorf tubes (or Reactivials™ for the 100° C test). Tubes were then incubated at each of four temperatures (15, 30, 60, and 100° C) for six days. Samples (5 $\mu$l) were removed from each tube at 0, 0.5, 1, 2, 4, 8, 24, 48, 72,

and 96 hours and spotted on a PDA plate. After drying, the plates were oversprayed with G. candidum, then incubated at room temperature overnight. Inhibition was recorded as plus or minus at each time and temperature.

pH Sensitivity

A 1-butanol crude extract (5 µl), at a concentration of 100 mg/ml, was dissolved in 200 µl of the treatment solution (Table 1). The concentration of each buffer was 0.01 M. Each buffer was adjusted to a pH equal to its $pK_a$ with 1 M sodium hydroxide or 1 M hydrochloric acid. The samples were incubated at room temperature for four days. At 0, 1, 2, 3, and 4 days, each solution (10 µl) was spotted onto PDA and bioassayed. All treatments were run in duplicate. The positive control was the 1-butanol extract dissolved in water and methanol. The negative controls were the buffers without the extract.

Table 1. Buffers for testing pH sensitivity of the toxin.

| Buffers | pH |
|---|---|
| 1. Citric Acid | 3.06 |
| 2. Citric Acid | 4.75 |
| 3. Ammonium Acetate | 4.75 |
| 4. Citric Acid | 5.40 |
| 5. Pipes | 6.80 |
| 6. Tris | 8.00 |
| 7. Tris | 9.00 |
| 8. Boric Acid | 10.00 |

Pseudomycin Stability in Various Solvents

A 1-butanol crude extract (5 µl at 33 µg/µl) was added to 100 µl of solvent. Each solvent (100 µl) alone was used for the negative control. The solutions were incubated at room temperature for 24 hours, at which time they were dried under nitrogen then resuspended in 15 µl of methanol and 10 µl were removed for bioassay.

The solvents tested were: 1-propanol, (50% and 100%); TFA, (1, 2, and 3 M); HCl, (0.5, 1, 2, and 4 M); 0.1% TFA, 10% acetonitrile; 0.1% TFA, 90% acetonitrile; 100% 1-butanol; 100% methanol; 100% water; and 0.1% TFA.

## Results and Discussion

### Bioassay

Before a protocol for toxin purification can be adopted, a good bioassay must be developed which is preferably both sensitive and quantitative. At least two methods are described in the literature for testing syringomycin; 1) antimycotic activity against *Rodotorula pilimanae*, and 2) antimycotic activity against *Geotrichum candidum*. Both organisms are fast growing and show good sensitivity to the phytotoxin. The overspray method utilizing *G. candidum* was the method of choice because of the availability of the organism. Fractions resulting from the various purification steps were spotted onto PDA (Appendix) and allowed to dry. The plate was oversprayed

with a sterile water suspension of the fungus then incubated overnight at room temperature. Presence of toxin was indicated by a clear zone of inhibition. Weak zones of inhibition, that eventually overgrew with mycelia, were considered negative.

Known amounts of crude extract were spotted for assay on separate PDA plates. Great variation in zone size was noted depending on the age of the plate and the amount the solution spread on the plate. In an attempt to reduce variation, the sample was applied to antibiotic filter discs which were then laid on the agar plate before overspraying. The toxin adsorbed to the disc, preventing diffusion into the agar plate. This method was not suitable for the assay. It was therefore inaccurate to quantitate the amount of toxin present according to zone size. Results in the bioassays were recorded as plus or minus.

Purification

Solvents used during the purification procedures could affect the activity of the toxin. To check for possible changes in activity, 165 $\mu$g of 1-butanol crude extract was added to 10 $\mu$l of the solvent to be tested. Each vial was incubated for 24 hours at room temperature then dried under nitrogen and resuspended in 15 $\mu$l of methanol. An aliquot (10 $\mu$l) was removed from each vial and bioassayed. Each solvent was tested without the crude extract as a negative

control. Pseudomycin remained active in all solvents tested except the 4 M HCl (Table 2). This may be due to either a low pH value or a high salt concentration resulting from the evaporation step. TFA, at 3 M, and the remaining concentrations of HCl, also decreased the zones of inhibition.

Table 2. Sensitivity of toxin to different solvents. (-) = no zone of inhibition (+) = a zone of inhibition < 1 cm. dia. ++ = a zone of inhibition > or = 1 cm. dia.

| Solvent | Treatment | Control |
|---|---|---|
| 100% 1-propanol | ++ | - |
| 50% 1-propanol | ++ | - |
| 3 M TFA | + | - |
| 2 M TFA | ++ | - |
| 1 M TFA | ++ | - |
| 0.5 M TFA | ++ | - |
| 0.1% TFA, 10% acetonitrile | ++ | - |
| 0.1% TFA, 90% acetonitrile | ++ | - |
| 0.5 M HCl | + | - |
| 1 M HCl | + | - |
| 2 M HCl | + | - |
| 4 M HCl | - | - |
| 100% 1-butanol | ++ | - |
| 100% methanol | ++ | - |
| 100% water | ++ | - |
| 100% water,0.1% TFA | ++ | - |

Heat stability was tested at four different temperatures; 15, 30, 60, and 100° C (Table 3). Butanol crude extract (75 $\mu$l), at a concentration of 33 $\mu$g/$\mu$l was incubated at each temperature for six days. Throughout the incubation period, 5 $\mu$l aliquots were removed and bioassayed. The toxin showed heat stability for up to four days when incubated at 15 or 30° C. When incubated at 60° C

there was total loss of activity by the fourth day. When the extract was incubated at 100° C there was a large decrease in the zone of inhibition for toxin heated 30 minutes and a complete loss of activity for toxin heated four hours. This toxin demonstrates considerable heat stability.

Table 3. Heat stability of the toxin. (+) = zones of inhibition < 2 cm. (++) = zones of inhibition > or = 2 cm. (-) = no apparent zone of inhibition.

| | Time (hours) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Temp. ° C | 0 | 0.5 | 1 | 2 | 4 | 8 | 24 | 48 | 72 | 96 |
| 15 | ++ | + | ++ | ++ | + | + | ++ | + | + | + |
| 30 | ++ | ++ | ++ | ++ | ++ | + | ++ | + | + | + |
| 60 | + | + | + | + | + | + | + | + | + | + |
| 100 | ++ | + | + | + | - | - | - | - | - | - |

Previous observations indicated the loss of toxin activity at pH values less than 6.5 (Dr. Avi Nachmias, unpublished). Partially purified pseudomycin (5 $\mu$l), at a concentration of 100 mg/ml, was dissolved in 200 $\mu$l of water, methanol (positive control), and each of eight different buffers (0.01 M). In addition, one tube of each treatment without the extract was prepared as a negative control. Each buffer was adjusted to a pH equal to its $pK_a$. Each solution was incubated at room temperature. After 0, 1, and 2 days, 10 $\mu$l of each solution was spotted onto PDA and bioassayed. Pseudomycin remained active in the water, methanol, and the buffers at or below a pH of 4.75 (Table 4). Activity in buffers at or above pH 5.4 was apparent

initially but the zones of inhibition were overgrown in two days. The static inhibition was also noted in the same control treatments and may be due to high pH values. These were recorded as negative results.

Table 4. Sensitivity of activity to pH changes.

| Treatments | pH |
|---|---|
| 1. Citric Acid | 3.06 |
| 2. Citric Acid | 4.75 |
| 3. Ammonium Acetate | 4.75 |
| 4. Citric Acid | 5.40 |
| 5. Pipes | 6.80 |
| 6. Tris | 8.00 |
| 7. Tris | 9.00 |
| 8. Boric Acid | 10.00 |

| | Results | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Day | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 0 | + | + | + | + | + | + | + | + |
| 1 | + | + | + | + | + | - | - | - |
| 2 | + | + | + | + | + | - | - | - |
| 3 | + | + | + | + | + | - | - | - |
| 4 | + | + | + | + | + | - | - | - |

Cultures of P. syringae MSU 16H were initially grown for six days in shake culture in modified Dyes media supplemented with ferric chloride, ornithine, and histidine (Appendix). Previous research indicated that ferric chloride at concentrations of 2 $\mu$M increased production of the toxin from P. syringae MSU 174 (11) and of syringomycin (9). Hemming (11) examined toxin production after the addition of individual amino acids. Both histidine and ornithine significantly increased toxin production.

Cells were removed from culture by centrifugation. To

determine if the majority of the toxin was extruded from the cells, pelleted cells were checked for toxin content. The cells were washed twice by suspending them in water, then centrifuging. Lysis was accomplished by first freezing at -70° C, then thawing, repeating the action twice. Second, the suspension was sonicated in a Kontes sonicator for 10 minutes. Cellular debris was resuspended in 5 ml of water, then 10 µl was spotted onto PDA and bioassayed as described above. No zones of inhibition were noted. All of the toxin appeared to have been extruded from the cells.

The culture supernatant liquid was extracted with 1-butanol. The toxin partitioned into the butanol phase which was subsequently flash evaporated to dryness then resuspended in methanol (Appendix, Figure 8). An average of 0.227 grams of crude extract was recovered per liter of culture (Table 5).

Table 5. Purification table for the butanol extraction procedure.

| Stage of Purification | grams dry weight per liter | purification fold |
|---|---|---|
| Centrifuge supernatant | 1.657 | 1 |
| Butanol extract | 0.227 | 7 |
| Amberlite XAD-2 | 0.0066 | 251 |
| RP-HPLC | 0.0002 | 8285 |

At a later date a more efficient purification protocol was adopted. This protocol was a slight modification of that published by Bidwai, et al. (2), for the purification

of syringomycin.

Cells were grown for six days in still culture in PDB at room temperature. The culture was mixed 1:1 (v/v) with acetone then centrifuged to remove cellular debris. The supernatant was first flash evaporated to 200 ml, then acetone was added to 60% (v/v). The mixture was allowed to precipitate overnight. Precipitate was removed by centrifugation. Toxin activity remained in the supernatant liquid. The supernatant liquid was concentrated, then resuspended in one liter of 0.1% trifluoroacetic acid (TFA), and loaded onto an Amberlite XAD-2 column (Appendix, Figure 9). After elution with a propanol gradient, the fractions were bioassayed.

Positive fractions were combined, filtered, and subjected to reverse phase high performance liquid chromatography (RP-HPLC). Two different solvent systems were utilized; 1) a 1-propanol nonlinear gradient, and 2) an acetonitrile linear gradient. Propanol has been recommended as the solvent of choice for the separation of peptides and proteins because the concentrations needed for elution are lower than with other organic modifiers, reducing the chance of activity loss. Acetonitrile is a good choice for the same reasons. TFA (0.1%) is necessary for adjustment of the pH (see Chapter III) and acts as an ion pairing agent. TFA modifies the polarity of the peptide through ion-pair formation which leads to an increase in retention time and

therefore, better separation (26,42). Volatility of TFA is also a positive aspect when considering amino acid analysis and sequencing.

The propanol gradient was a nonlinear (Waters #2) gradient of 0 to 30% 1-propanol, 0.1% TFA over 35 minutes. Each peak from the propanol gradient was collected, concentrated by flash evaporation, then tested for bioactivity. Peaks 3, 4, and 6 were found to be active (Figure 1). The eluate collected from 16 to 20 minutes (labeled #5 in Figure 1) was also active but no definite peak was seen on the elution profile. The retention times are listed in Table 6. These peaks were reinjected for further purification.

Table 6. Retention times of peaks from the HPLC after elution with a nonlinear propanol gradient.

| Peak number | retention time |
|---|---|
| 3 | 13 min. 30 sec. |
| 4 | 14 min. 45 sec. |
| 5 | ---------- |
| 6 | 22 min. 0 sec. |

The final step was to subject the eluate from peak #4 to the RP-HPLC with the linear acetonitrile gradient, 0 to 80% acetonitrile over 20 minutes. The retention time was 14 minutes and 45 seconds (Figure 2). This procedure yielded an average of 0.0015 grams of purified toxin as compared to 0.0002 grams with the butanol procedure (Tables 5 and 7).

Figure 1. RP-HPLC elution profile of partially purified pseudomycin from the Amberlite XAD-2 column.

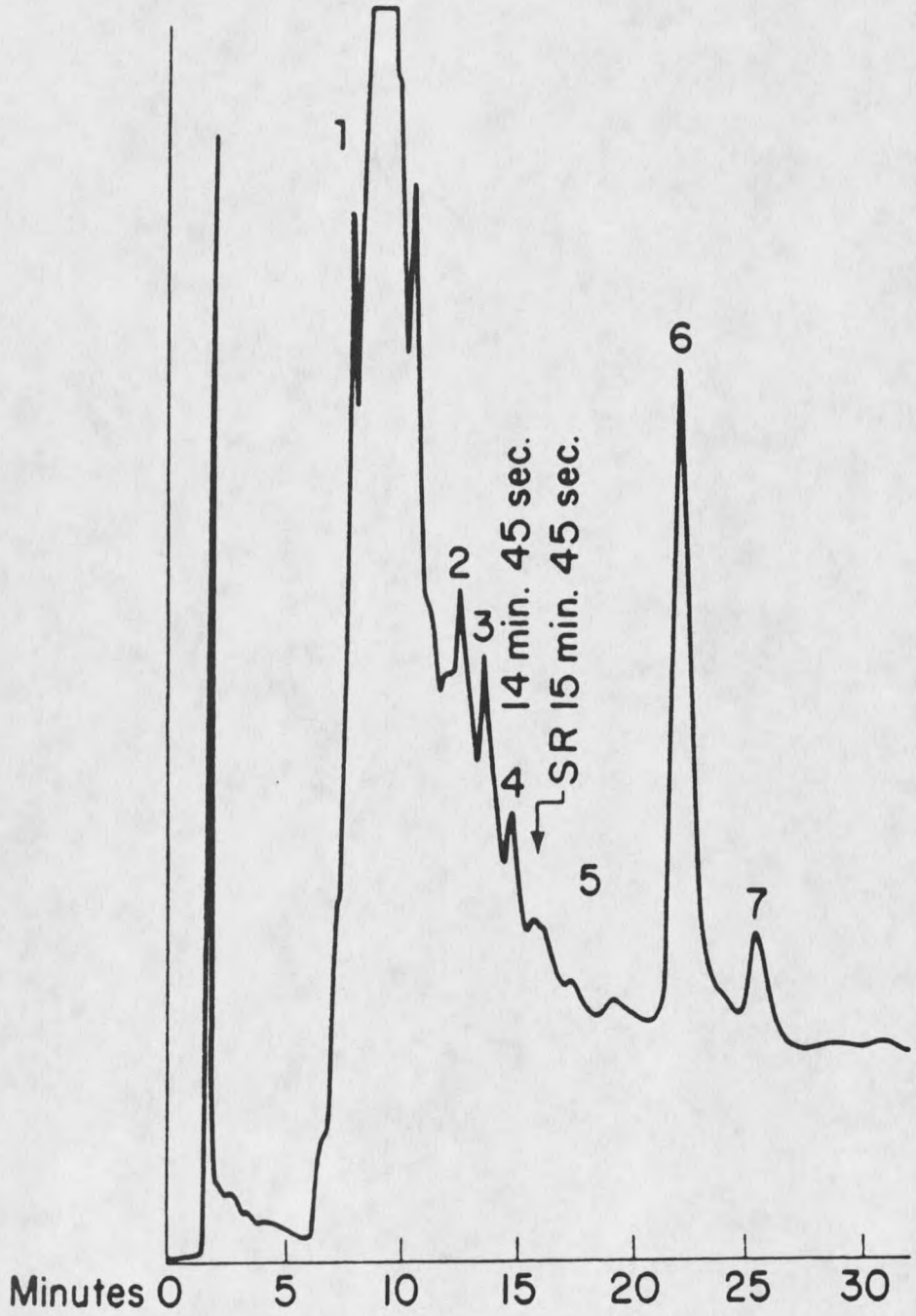
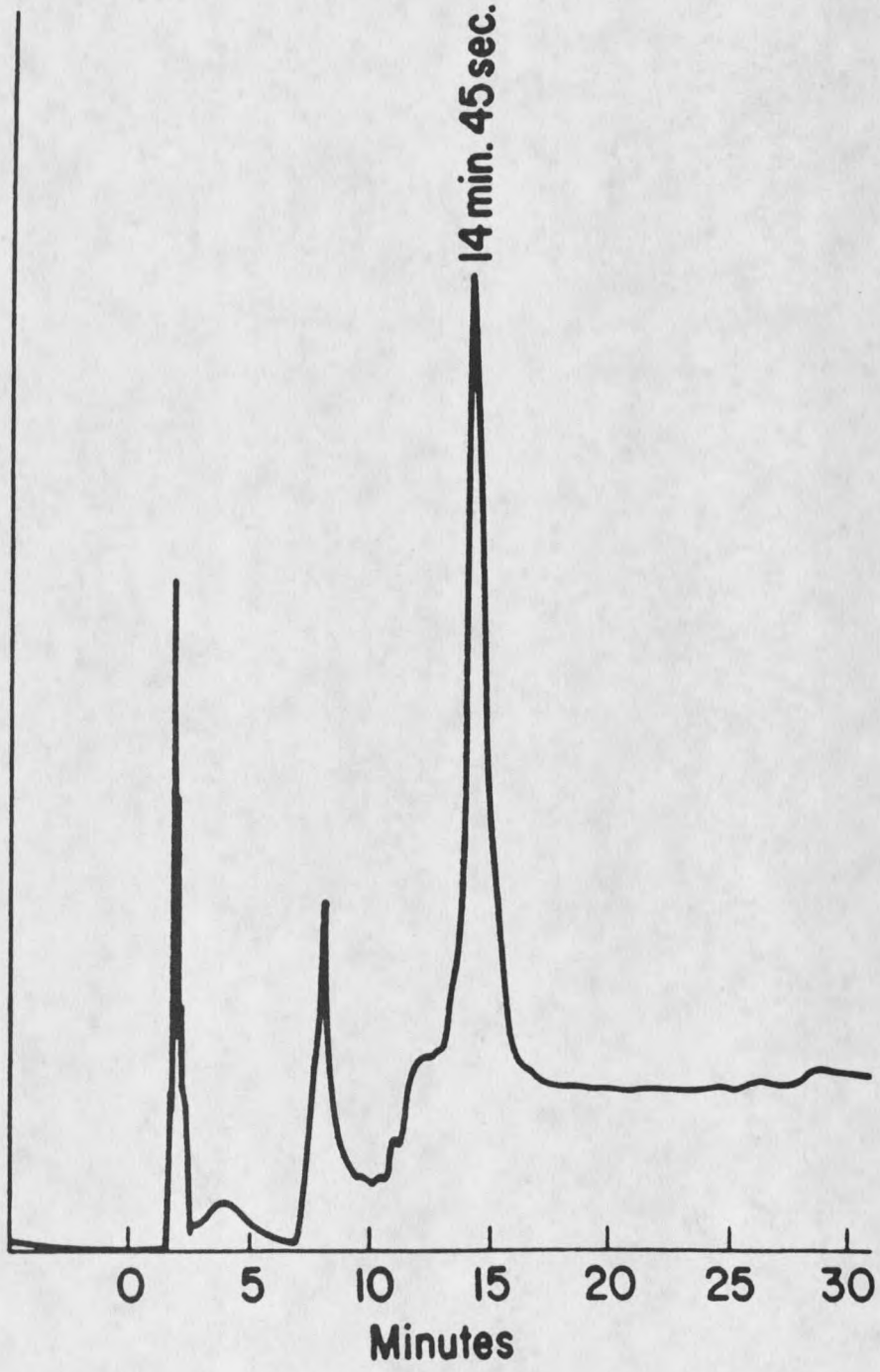


Figure 2. RP-HPLC elution profile of pseudomycin run on a linear gradient of 0-80% acetonitrile over 20 minutes.



Both the 1-butanol extraction method and the acetone precipitation method resulted in the isolation of the same compound as confirmed by thin layer chromatography and mass spectrometry data.

Table 7. Purification table for the acetone precipitation procedure.

| Stage of Purification | Grams of Dry Weight per liter | Purification Fold |
|---|---|---|
| PDA culture | 27.6 | 1 |
| 1st acetone ppt. | 15.0 | 1.8 |
| 2nd acetone ppt. | 12.8 | 2.2 |
| Amberlite XAD-2 | 0.016 | 1,725 |
| RP-HPLC | 0.0015 | 18,400 |

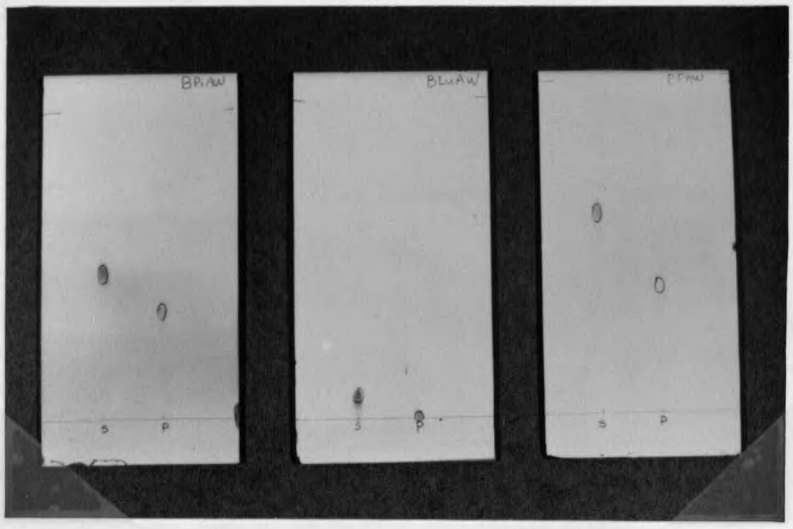
As a preliminary check, the eluate collected at the time of each peak from the propanol gradient, was subjected to FAB mass spectrometry. HPLC peak number 4 was the only peak that appeared to be pure, having a single mass ion peak. Mass spectra data from 3 and 5 had more than one peak, one of which had the same mass ion assignment as the peak from HPLC peak number 4. Spectral data for HPLC peak number 6 also showed several peaks but none at the same mass as HPLC peak number 4. This may suggest a second antimycotic compound in these preparations.

PDB alone was carried through the same purification steps as the P. syringae culture. There were no detectable toxins present in PDB alone.

Confirmation of Purity

Purity is mandatory for the proper identification of an unknown compound. A single peak upon elution from the HPLC column is suggestive of purity but in itself is not enough. Thin layer chromatography (TLC) is one method for examining purity. Peptides are markedly hydrophilic compounds and are only slightly soluble in nonaqueous solvents. Solvent systems used in their separation must generally contain water. Butanol: formic acid: water (75:15:10) was first utilized as suggested by Brenner (3). This solvent system was inadequate because it did not substantially move the compound away from the origin. Butanol:pyridine:acetic acid:water (15:10:3:12), was the second solvent of choice. The RP-HPLC sample (3 $\mu$g) was spotted on to a silica gel TLC plate. Syringomycin was also spotted on the plate for a comparative standard. Both compounds migrated as single spots with $R_f$'s of .59 and .37, respectively (Figure 3). Not only did pseudomycin appear pure, but it migrated differently than syringomycin. To further establish purity, pseudomycin was run in two additional solvent systems; picoline or lutidine was substituted for pyridine. These solvents are methylated analogs of pyridine. Again single spots were apparent with $R_f$ values as indicated in Figure 3.

Figure 3. Thin layer chromatography of pseudomycin.



Chemical Characterization

The purified sample was subjected to Fast Atom Bombardment (FAB) mass spectrometry. Mass spectrometry utilizing electron bombardment for peptides requires chemical degradation and derivatization, while FAB mass spectrometry makes it possible to analyze underivatized large polar biomolecules (12). The sample was run in two different matrices, glycerol and thioglycerol. The mass H+ was found to be 1207 (Figure 4). The same sample was used for amino acid analysis and sequencing. Preliminary results for pseudomycin indicate the presence of seven amino acids; aspartate, serine, lysine, phenylalanine, and arginine (1:1:1:3:1) and one unknown amino acid. The amino acid analysis of syringomycin confirmed published data; arginine, phenylalanine, serine, and 2,4-diaminobutyric acid (1:1:2:2).