ISOLATION AND PARTIAL CHEMICAL CHARACTERIZATION OF AN ANTIMICROBIAL PEPTIDE PRODUCED BY *BACILLUS SUBTILIS*

by

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Abstract

An endophytic bacterium (*Bacillus subtilis* EN 63-1) isolated from apple fruit produces an antibiotic which has been purified to homogeneity and partially characterized. It consists of an acidic peptide containing asparagine/aspartate, glutamine/glutamate, serine, glycine, alanine, proline, valine, and leucine in a ratio of 2:3:1:1:1:1:1:4. Based on its infrared spectrum, the peptide antibiotic contains an acyl chain and has a lactone bond in its structure. Its molecular weight, estimated by SDS-polyacrylamide gel electrophoresis, is 1.5 kDa; however, it forms aggregates in excess of 20 kDa. The peptide antibiotic is similar to a number of lipopeptide antibiotics often termed "biosurfactants" and classified as fatty acid containing peptolides.

The antibiotic has a broad spectrum of antimicrobial activity against Gram negative bacteria, shows little activity against Gram positive bacteria, and displays some anti-fungal activity. Studies on the effect of the peptide on *Agrobacterium vitis* cells suggest a bactericidal mode of action rather than a bacteriostatic effect; however, cell death is not associated with cell lysis.

Antimicrobial activity is associated with the active growth of the producing organism rather than with the stationary phase. The activity of the peptide antibiotic is stable over a fairly wide range of pH and temperature. Peptide activity begins to decrease at alkaline pH, but is stable at neutral and acidic pH. Complete loss of activity is observed above pH 10. At an acidic pH (pH 3.0) the peptide begins to lose activity above 60°C, demonstrating a complete loss of activity at 100°C; however, at neutral pH the peptide remains stable up to 80°C and a complete loss of activity is not observed at 100°C.

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I. Introduction

Many types of antibiotics are produced by a wide assortment of microorganisms. Over 8,000 antibiotics are known to exist and hundreds more are discovered yearly (Brock and Madigan, 1991, pg. 361); however, few ever 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, pg. 361), antibiotics are produced in excess of 100,000 tons per year with annual gross sales amounting to nearly 5 billion dollars.

A number of chemically related antibiotics exist that 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 chemistry 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:

- generally much smaller than proteins
- generally a family of closely related peptides rather than a single substance is produced by an organism
- often contain constituents other than amino acids
- often contain unique amino acids and 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. The majority of the antibiotics produced by *Bacillus* are peptides (Korzybski et al., 1978, pg. 1529). Of all the antibiotics produced by *Bacillus*, most are active against Gram positive organisms; however, compounds such as polymyxin, colistin, and circulin exhibit

activity mainly against Gram negative organisms (Katz and Demain, 1977). Some of the peptides produced by *Bacillus* (mainly the lipopeptides) demonstrate interesting 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* demonstrate not only antimicrobial properties, but are excellent surfactants as well. Jenny et al. (1991) suggested 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 such as agriculture, the food industry, and even research. In some cases peptide antibiotics which demonstrate surface active properties (biosurfactants) may even be useful to the petroleum industry (Fiechter, 1992).

In a study aimed at using *Bacillus* spp. 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 produced some type of extracellular inhibitory compound. The isolation, purification, and characterization (chemical and physical) of this antimicrobial agent is presented in this thesis.

II. Literature Review

A. Classification of Peptide Antibiotics

1. Problems With Classification

There are many possible methods for the classification of antibiotics, yet it remains a very difficult task to classify and organize them all using any one method. More than 8,000 antibiotics are known to exist (Brock and Madigan, 1991, pg. 361), and the numbers increase every year as new antibiotics are discovered. Some of the methods available for classification of antibiotics include classification according to origin, classification according to biosynthesis, and classification according to mode of action.

Classification according to origin provides a method by which all the antibiotics can easily be arranged into many large groups based on the organisms that produce them; however, there is little possibility for further rational subdivision within these groups (Berdy, 1974). Organisms can produce antibiotics of diverse chemical structure, having equally diverse properties, thus making it very difficult to further categorize antibiotics classified according to origin. Although most of the antibiotics produced by members of the genus *Bacillus* are peptides, antibiotics belonging to other chemical classes exist (Katz and Demain, 1977). A good example is the aminoglycoside antibiotic butirosin, which is produced by *Bacillus circulans* (Anderson et al., 1972). Further complicating this classification scheme is the fact that very similar antibiotics can be produced by completely different organisms. Nisin and subtilin are two lanthionine containing antibiotics (lanthionines) that differ only in a few amino acids, yet nisin is produced by lactic streptococci and subtilin is produced by *Bacillus subtilis* (Hurst, 1981).

Classification according to biosynthesis offers a classification scheme with some potential; however, such a classification scheme is not feasible to date because it is limited to the relatively few antibiotics where the method of biosynthesis has been determined (Berdy, 1974). In general, classification according to biosynthesis is strongly related to classification according to structure. In other words, antibiotics with similar structures are generally produced by similar pathways. An exception to this rule is the peptide antibiotics. Some peptide antibiotics are produced ribosomally with post translational modifications, while other peptide antibiotics are a result of enzyme pathways. Nisin is an example of a ribosomally produced antibiotic (Hurst, 1981), while gramicidin is an example of an antibiotic produced through an enzyme pathway (Katz and Demain, 1977).

Antibiotics may be classified according to their mode of action. This method characterizes antibiotics on the basis of how they affect sensitive organisms, and to date is limited to relatively few antibiotics. Only those antibiotics where the mode of action has been determined can be classified in this manner (Berdy, 1974). Some antibiotics may fall into more than one classification using such a system because some antibiotics can demonstrate more than one mode of activity. For example, gramicidin S affects both membrane function as well as oxidative phosphorylation (Berdy, 1974). Other antibiotics can have different modes of action on different bacteria. Plantaracin C, a peptide antibiotic produced by *Lactobacillus plantarum*, demonstrates only bacteriostatic activity against *Lactobacillus sake*, but shows lytic activity towards *Lactobacillus fermentum* (Gonzalez et al., 1994).

2. Classification of Peptide Antibiotics According to Chemical Structure

Classification according to chemical structure is probably the least ambiguous and most ordered method available for the classification of antibiotics. The technique allows for a logical ordering of the compounds into families, subfamilies, groups, and classes based on their chemical structure. Berdy (1974) offers a classification based on chemical criteria that divides antibiotics into 10 major families. Antibiotics are separated into different families based on the moiety of the molecule responsible for antibiotic activity. Within the 10 families, the various antibiotics are arranged into subgroups according to the individual characteristics of the antibiotics (size, biological activity, homogeneity, etc.).

Table 2.1: Key to the main antibiotic families

Family Number	Main Chemical Feature
1	Carbohydrate antibiotics
2	Macrocyclic lactone antibiotics
3	Quinone and similar antibiotics
4	Amino acid, peptide antibiotics
5	Nitrogen-containing heterocyclic antibiotics
6	Oxygen-containing heterocyclic antibiotics
7	Alicyclic antibiotics
8	Aromatic antibiotics
9	Aliphatic antibiotics
10	Miscellaneous antibiotics

Adapted from Berdy (1974)

Table 2.1 shows that peptide antibiotics represent a single family of antibiotics. As with all of the other antibiotic families, the peptide antibiotics can be further divided into subfamilies, and the subfamilies into groups. Table 2.2 details the chemical

Table 2.2: Classification of the peptide antibiotics

Subfamily	Group	Example	Reference
Amino acid derivatives	Antibiotics derived from amino acids	Penicillin	Korzybski et al., 1978, pg. 1663
	Diketopiperazine derivatives	Aranotin	Nagarajan et al., 1968
Homopeptides	Oligopeptides	Distamycin	Arcamone et al., 1964
	Linear homopeptides	Edeine	Hettinger and Craig, 1970
	Cyclic homopeptides	Bacitracin	Craig et al., 1952
Heteromer peptides	Cyclopeptides with fatty acids	Polymyxin	Katz and Demain, 1977
	Cyclopeptides with sulfur containing heterocycles	Bottromycin	Nakamura et al., 1965
	Chelate forming peptides	Albomycin	Turkova et al., 1963
[@]Peptolides	Chromopeptolides	Actinomycin	Katz and Pugh, 1961
	Peptolides with fatty acids	Surfactin	Arima et al., 1968
	Peptolides with N containing heterocycles	Virginiamycin	Crooy and De Neys, 1972
	Peptolides without other constituents	Telomycin	Sheehan et al., 1963
High molecular weight peptides	Polypeptides	Nisin	Gross and Morell, 1971

Adapted from Berdy (1974)
[@]Peptolides are cyclic antibiotics containing a lactone bond

characterization of the peptide antibiotics according to Berdy (1974). The classification scheme (Table 2.2) allows for the differentiation of the peptide antibiotics in a very ordered and logical manner. Such a system is very flexible because new divisions can be created at any point in the classification scheme to allow for new compounds that may not fit in the existing framework. Another advantage to this system is that antibiotics sufficiently characterized by physical, chemical, or biological data may be classified prior to the elucidation of structure.

Although it is quite dated, Berdy's classification system (1974) described above is still very functional, due primarily to its flexibility. Betina (1983, pg. 117) describes the classification system of Berdy (1974), and states that it is a consistently elaborated open classification scheme. This same system was used by Brock and Madigan (1991, pg. 361). Classification of antibiotics according to chemical structure still appears to be the best system for categorizing antibiotics.

B. Chemical Characterization of Peptide Antibiotics

Peptide antibiotics cannot be described using a single definition because they are very diverse in terms of chemical structure and physical characteristics. Perhaps the only common feature of all peptide antibiotics is the presence of amino acid(s). The peptide antibiotics are better described using a number of general properties that distinguish them from proteins and other classes of antibiotics.

1. Molecular Weight

According to Katz and Demain (1977), peptide antibiotics can range from as small as 270 Daltons (bacilysin) to as large as 4500 Daltons (licheniformins). Generally speaking, the molecular weight of peptide antibiotics is far less than most proteins, and greater than most other types of antibiotics. Some peptide antibiotics can form aggregates with molecular weights exceeding 100,000 Daltons. Lactacin F, a bacteriocin produced by *Lactobacillus acidophilus*, forms micellar-like globular particles with a molecular weight of approximately 180,000 Daltons (Muriana and Klaenhammer, 1991).

2. Structure

a. Unique amino acids

Peptide antibiotics contain many amino acids in their structure that are unique to compounds of this type (Katz and Demain, 1977). These amino acids are not found in proteins and include D-amino acids, β-amino acids, basic amino acids, methylated amino acids, imino acids, and sulphur containing amino acids (Katz, 1971). Specific examples of these unique amino acids are listed in Table 2.3.

b. Other components

Many peptide antibiotics are composed entirely of amino acids. Examples include gramicidin, mycobacillin, and tyrocidine. Other peptide antibiotics have a peptide backbone, but also contain components such as fatty acids, amino sugars, hydroxy acids,

Table 2.3: Some unusual amino acids found in peptide antibiotics

Unusual Amino Acid	Antibiotic	Reference
diaminobutyric acid	polymyxin	Katz and Demain, 1977
dehydrobutyrine	nisin	Hurst, 1981
ornithine	bacitracin	Katz and Demain, 1977
lanthionine	nisin	Hurst, 1981
diaminopropionic acid	edeine	Katz and Demain, 1977
dehydroalanine	nisin	Hurst, 1981

Adapted from Bodanszky and Perlman (1969)

amines, and N-containing heterocycles (Katz, 1971). Polymyxin B is a decapeptide in which residues 4 through 10 form a ring, leaving a tripeptide tail which is bound by an amide linkage to a branched chain fatty acid (Sadoff, 1971). The antibiotic edeine A is a strongly basic, linear oligopeptide which contains the biogenic amine spermidine (Katz and Demain, 1977). Edeine B is the same as edeine A; however, guanylspermidine replaces spermidine in the former (Sadoff, 1971).

c. Structural disparity

According to Katz (1971) microorganisms usually produce a family of related antibiotics rather than a specific compound. In most cases the structural differences between a family of related compounds produced by an organism are limited to only slight differences in the overall structure of the compound. Acylpeptides (APD I, II, and III) produced by *Bacillus subtilis* (Hozono and Suzuki, 1983) have identical amino acid compositions, but differ in the length of their fatty acid residues. Tyrocidines A and B are

cyclic decapeptides that differ from each other by the substitution of L-tryptophan for L-phenylalanine at position six (Sadoff, 1971).

4. Other Properties

a. Resistance to proteolytic enzymes

Peptide antibiotics are generally resistant to hydrolysis by peptidases and proteases of plant and animal origin (Bodanszky and Perlman, 1964). This is a very general rule and exceptions exist including gramicidin S which is susceptible to hydrolysis by subtilopeptidase A (Yukioka et al., 1966) and polymyxin B which is susceptible to the enzymes ficin and papain (Paulus and Gray, 1964). Some of the larger peptide antibiotics, often termed bacteriocins, appear to be more susceptible to enzymatic attack. Lactacin F (Muriana and Klaenhammer, 1991) is susceptible to hydrolysis by ficin, trypsin, proteinase K, and subtilisin, while a bacteriocin produced by *Staphylococcus aureus* (Gagliano and Hinsdill, 1970) was susceptible to two of the three enzymes tested. It is not surprising that some of the larger peptide antibiotics which contain more hydrolysis sites and fewer modified amino acids are more susceptible to proteolytic attack.

b. Surface activity

Some of the peptide antibiotics demonstrate unique properties that are often overlooked in light of their antimicrobial properties. Certain acylpeptides demonstrate both antimicrobial and surface active properties (i.e. they lower the surface tension of water). A mixture of four closely related lipopeptides isolated from *Bacillus licheniformis*

(Jenny et al., 1991) decreased the surface tension of water from 72 mN/m to 27 mN/m at a concentration of 15 mg/ml. The same compounds demonstrated antibiotic activity against bacteria and yeast. Some other peptide antibiotics that demonstrate surface activity include surfactin (Arima et al., 1968), and arthrofactin (Morikawa et al., 1993).

c. Cyclic structures

A number of peptide antibiotics do not have a free carboxyl or amino terminal in their structure because many of the peptide antibiotics are cyclic. Cyclization can occur both on the entire structure and on small areas within the macrocyclic structure (Bodanszky and Perlman, 1969). Bacitracin (Figure 2.1) contains a thiazoline ring within its structure created by a condensation of cysteine and isoleucine; it also contains a cyclic hexapeptide created by an amide linkage between the β-carboxyl group of aspartic acid and the epsilon amino group of lysine (Weinberg, 1967). Nisin (Figure 2.2) contains four internal ring structures each created by a sulphide bridge between aminobutyric acid and alanine, and one internal ring structure created by a sulphide bridge between two alanine residues (Hurst, 1981). Cyclization in peptide antibiotics can also result from a lactone linkage. Surfactin (Figure 2.3) has a cyclic structure consisting of seven amino acids covalently bonded at one end to the carboxyl function and at the other end to the hydroxyl function of a β-hydroxy fatty acid by a lactone bond (Cooper and Zajic, 1980).

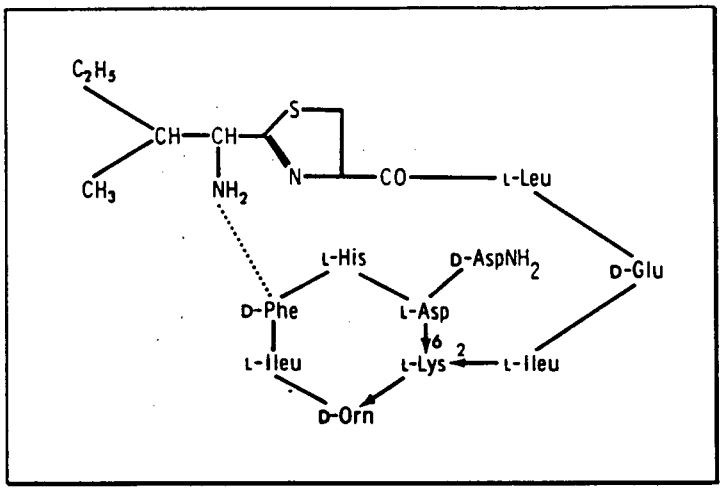


Figure 2.1: Structure of bacitracin A.
(Katz and Demain, 1977)

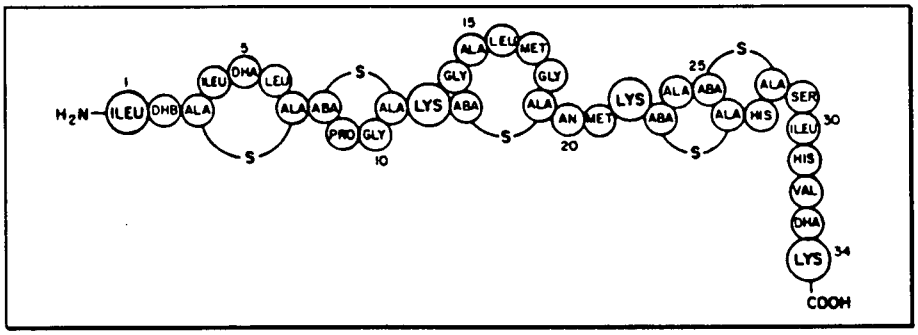


Figure 2.2: The structure of nisin (Hurst, 1981) ABA = aminobutyric acid, DHB = dehydrobutyrine, DHA = dehydroalanine, Ala-S-Ala = lanthionine, Ala-S-ABA = β-methyllanthionine

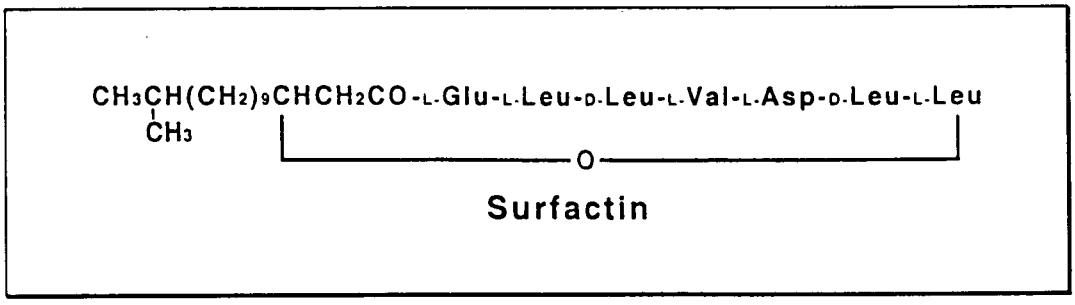


Figure 2.3: Structure of surfactin (Morikawa et al., 1993)

C. Biosynthesis of Peptide Antibiotics

Biosynthetic pathways have been determined for relatively few of the peptide antibiotics discovered. Peptide antibiotics can be divided into two main groups based on how they are biosynthesized. The peptides are either a result of a multi-enzyme pathway, or they are produced ribosomally.

1. Enzymatic Production of Peptide Antibiotics

Marahiel (1992), Kleinkauf and Dohren (1990), Katz and Demain (1977), Kurahashi (1974), and Perlman and Bodanszky (1971) reviewed the biogenesis of peptide antibiotics. The mechanism for the formation of the classical low molecular weight peptide antibiotics differs markedly from that of protein synthesis (Katz, 1971). A protein thio-template pathway is involved in the biosynthesis of gramicidin, tyrocidine, surfactin, bacitracin, enniatin, actinomycin, cyclosporin, and the first step of β-lactam synthesis (Marahiel, 1992). Although the enzymes involved in the production of different peptide antibiotics are not the same, the mechanisms involved are similar (Bodanszky and Perlman, 1969). Rather than presenting the biosynthesis of each peptide antibiotic individually, the biosynthesis of peptide antibiotics will be presented using a unified picture.

a. Production in the absence of protein synthesis

A large number of peptide antibiotics appear in the culture broth of the producing organism after active macromolecular synthesis and rapid growth (Katz, 1971). Some

authors (Sadoff, 1971; Hodgson, 1970) have attempted to link peptide antibiotics with sporulation of the producing cell. Peptide antibiotic synthesis appears to be unrelated to protein synthesis. Certain inhibitors of protein and nucleic acid synthesis have little or no effect on the production of peptide antibiotics. Gramicidin S continues to accumulate in *Bacillus brevis* fermentations when growth and protein synthesis have been terminated with 5'-fluorouracil, 5-bromouridine, puromycin, mitomycin C, or chloramphenicol (Katz et al., 1965). Similar effects have been demonstrated with bacitracin, tyrocidine, and polymyxin (Katz, 1971). Certain inhibitors of protein and nucleic acid synthesis can in fact stimulate the incorporation of particular amino acids into peptide antibiotics (Katz, 1971). The cessation of protein synthesis makes it easier for peptide antibiotics to compete for the availability of amino acids in the amino acid pool.

The addition of certain amino acids to the culture media can direct the biosynthesis of the antibiotic formed, such that it will be rich in that particular amino acid (Katz and Demain, 1977). This suggests that the multi-enzyme complexes involved in the biosynthesis of peptide antibiotics demonstrate a lack of specificity which leads to the substitution of structurally analogous amino acids at certain positions in the peptide chain. This type of broad range specificity is not seen amongst proteins and leads to a broad spectrum of antibiotic analogs produced by a single organism. Single amino acid substitutions observed in proteins are the result of changes in the nucleotide sequence of the structural genes rather than environmental or metabolic changes (Katz, 1971).

b. Cell free synthesis of peptide antibiotics

Cell free synthesis of many peptide antibiotics including gramicidin S (Tomino et al., 1967), tyrocidine (Roskoski et al., 1970), and edeine (Kurylo-Borowska, 1967) has been achieved. Cell free synthesis generally requires ATP, Mg^{2+}, a reducing agent, the requisite amino acids, and the particle free supernatant fluid which contains the enzyme complexes (Betina, 1983, pg. 248).

Marahiel (1992) offered a generalized outline of the non-ribosomal synthesis of peptide antibiotics. The first step in peptide antibiotic biosynthesis involves activation of the amino acids as acyl adenylates. ATP serves as the energy source and Mg^{2+} is required. The amino-acyl adenylates are covalently linked to peptide synthetases by a carboxy thioester bond to form an enzyme bound aminoacyl adenylate complex (Figure 2.4). Peptide synthetases that activate more than one amino acid bind the individual amino acids at different sites on the multi enzyme complex. The amino acids are bound in the same order that they appear in the peptide chain, thus the multi-enzyme complex serves as the template for the pre-defined peptide sequence. The elongation reaction is catalyzed by an enzyme bound cofactor known as a 4'-phosphopantetheine. The activated amino acids are transferred to the thiol group of the cofactor (trans-thiolation reaction) which serves as an internal transport system. Repeated trans-peptidation and trans-thiolation reactions are responsible for peptide chain elongation. Chain elongation is presumably completed by cyclization of the peptide antibiotic or by the action of a specific thioesterase.

The growth of peptide antibiotics produced non-ribosomally occurs at the carboxyl end leaving the amino terminal in either a free, formylated, or acylated state. The first

possibility is most likely if subsequent cyclization is to occur; however, the latter two possibilities may apply in the case of linear peptides or branched cyclic structures (Kleinkauf and Dohren, 1990).

In the enzymatic formation of peptide antibiotics, linear precursor peptides may be modified in a number of ways. Modifications can occur at the peptide level (i.e. cyclization reactions) or at the amino acid precursor level preceding incorporation into the peptide chain (Kleinkauf and Dohren, 1990). Amino acid precursors can be methylated or epimerized; cyclization reactions include direct amide linkage of α-carboxyl with α-amino groups, lactonization, and amide linkage of α-carboxyls with β,γ, or δ-amino acids (Kleinkauf and Dohren, 1990).

Kurahashi (1981) proposed that there is a limit to the size of the multienzymes capable of carrying out peptide antibiotic synthesis, hence the limit for the synthesis of peptides by the multi-enzyme systems is approximately 25 amino acids. In fact, the largest peptide which is considered to be formed by a multienzyme complex is alamethicin which has 19 amino acid residues (Rindfleisch and Kleinkauf, 1976).

2. Ribosomal synthesis of peptide antibiotics

The larger peptide antibiotics, often referred to as bacteriocins, are not produced by enzyme pathways. The production of bacteriocins is generally sensitive to inhibitors that affect protein synthesis at the ribosomal level (Hurst, 1981). Generally speaking, bacteriocins are biosynthesized in the same manner as proteins. A good example is nisin which is ribosomally synthesized as a precursor peptide and then enzymatically modified (Jack et al., 1995).

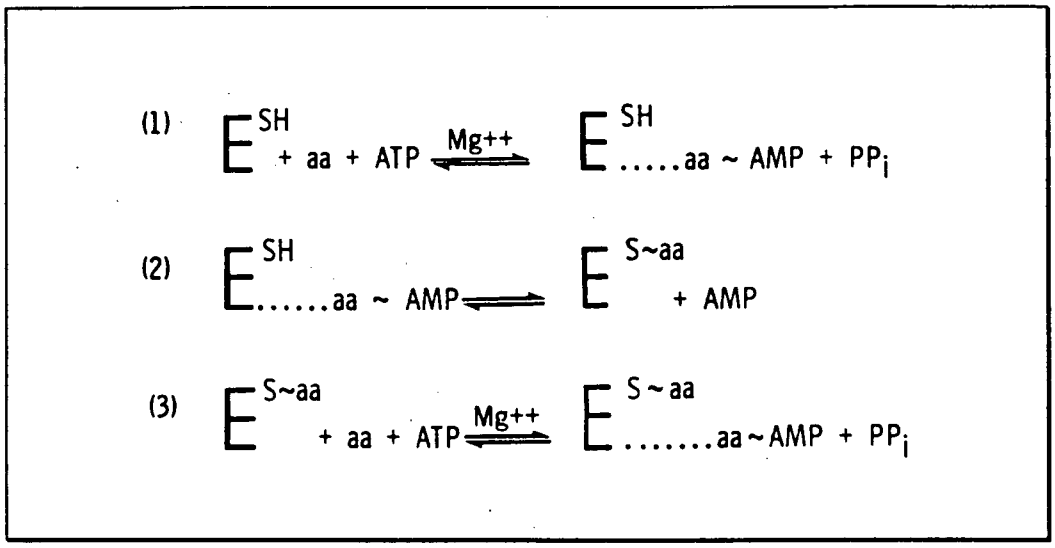


Figure 2.4: Mechanism for the activation of amino acids in peptide antibiotic synthesis (Lipmann, 1971).

The direct result of mRNA translation is a prepeptide molecule that has no biological activity. In many cases the prepeptide contains a propeptide domain at its C-terminal (Jack et al., 1995). The prepeptide chain goes through a series of enzymatic modifications that yield the mature antimicrobial structure (Schnell et al., 1988). The post-translational modifications are responsible for removing the C-terminal propeptide and creating the non-protein amino acids found in many bacteriocins. An example of a post-translational modification is the production of the α,β-unsaturated amino acids (didehydroalanine and didehydrobutyrine) in nisin by the dehydration of serine and threonine (Jack et al., 1995).

D. Mode of Action of Peptide Antibiotics

Peptide antibiotics are very diverse in form and function, therefore it seems

reasonable that their mode of action is not limited to a specific target in the cell. The only common feature in the mode of action among all peptide antibiotics is that they must gain entrance into the cell. The main targets of most antibiotics lie in the cytoplasmic membrane or within the cytoplasm, thus the antibiotic must penetrate through the outer layers of the cell envelope in order to be effective (Betina, 1983, pg. 290). The specific target involved in the mode of action of peptide antibiotics can vary considerably and is dependent upon the antibiotic itself, its concentration, and the organism involved. It can often be difficult to distinguish the primary effects of an antibiotic from the secondary effects that may be a result of growth inhibition (Storm and Toscano, 1979). Bearing these points in mind, the targets of antibiotic activity may include peptidoglycan synthesis, membrane function, nucleic acid synthesis, and protein synthesis.

1. Inhibitors of Cell Wall Synthesis

The prokaryotic cell wall is made up of peptidoglycan, teichoic acids, teichuronic acids, and lipopolysaccharides. The peptidoglycan lends rigidity and strength, and is the fundamental component of most bacterial cell walls (Brock and Madigan, 1991, pg. 57). In Gram positive bacteria it is present throughout the entire width of the cell wall in a complex with polysaccharides and teichoic acids. In Gram negative bacteria the peptidoglycan is present as a thin strip in the inner layer (periplasm) of a cell wall that is enclosed by an inner and outer membrane made up of lipopolysaccharide.

According to Brock and Madigan (1991, pg. 57), the basic backbone of peptidoglycan is composed of two alternating amino sugars, N-acetylglucosamine

(GlcNAc) and N-acetylmuramic acid (MurNAc). The latter is substituted with a short peptide chain consisting of L-alanine, D-alanine, D-glutamic acid, and either lysine (Gram positive organisms) or diaminopimelic acid (Gram negative organisms). In Gram negative bacteria some of the peptide chains on MurNAc are cross-linked to each other by a peptide bond between diaminopimelic acid and the terminal alanine residue. In Gram positive organisms the peptide chains on MurNAc are cross-linked by a pentaglycine interbridge.

Synthesis of the peptidoglycan can be divided into three stages (Betina, 1983, pg. 356). The first stage involves synthesis of the precursors UDP-GlcNAc and UDP-MurNAc-pentapeptide in the cytoplasm. The second stage requires transfer of the non-nucleotide portion of the precursors to the growing peptidoglycan, and the third stage involves cross-linking of the linear peptidoglycan strands by transpeptidation. Peptide antibiotics can interfere with the process at any of the three stages. Some of the peptide antibiotics that interfere with cell wall synthesis include bacitracin, vancomycin, amphomycin, and enduracidin.

Bacitracin is a mixture of closely related polypeptides that are active mainly against Gram positive organisms (Korzybski et al., 1978, pg. 1556). Three distinct modes of action have been determined for bacitracin A (Weinberg, 1967). The peptide can inhibit protein synthesis, it can interfere with peptidoglycan synthesis, and it can impair cell membrane function. It is difficult to distinguish the primary effects of the antibiotic; however, cell membrane function and peptidoglycan synthesis appear to be most sensitive to the antibiotic (Storm and Toscano, 1979). In terms of the peptidoglycan synthesis,

bacitracin appears to interfere with an enzyme responsible for the dephosphorylation of a lipid pyrophosphate involved in cell wall synthesis (Kucers and Bennett, 1987, pg. 751). The lipid pyrophosphate is an essential carrier compound involved in the second stage of peptidoglycan synthesis.

Vancomycin, a glycopeptide antibiotic (Sheldrick et al., 1978), also interferes with the second stage of peptidoglycan synthesis. Vancomycin forms a complex with a precursor of peptidoglycan synthesis and interferes with transfer of the peptidoglycan unit to the growing acceptor chain (Kucers and Bennett, 1987, pg. 1045).

2. Inhibitors of Cytoplasmic Membrane Function

The cytoplasmic membrane consists of a bilayer of phospholipids whose hydrophilic heads form the outer and inner membrane surfaces with the hydrophobic tails meeting at the center of the membrane (Brock and Madigan, 1991, pg. 48). Proteins invaginate the membrane and can be found at or near the membrane surface or they may penetrate the membrane. The cytoplasmic membrane forms a solubility barrier for both metabolites and ions. Proteins embedded in the membrane enable the cell to extrude or accumulate specific ions and maintain a constant cellular pH (Bakker, 1979). Peptide antibiotics can disrupt the membrane function in one of three ways: they may act as mobile ion carriers, they may form membrane channels, or they may cause a general disruption of membrane function.

Valinomycin is a good example of a peptide antibiotic that acts as a mobile ion carrier. According to Betina (1983, pg. 385), valinomycin sorbs into the membrane

surface and binds potassium ions from the aqueous phase. Once valinomycin has formed a complex with potassium, it diffuses to the other side of the membrane and dissociates within the cell, releasing potassium. Valinomycin binds potassium specifically and the transfer of potassium ions across the cell membrane is dependent on the concentration gradient. Transfer of potassium ions to the inside of the bacterial cell can disrupt membrane potential to the point where it is too low to drive ATP synthesis (Bakker, 1979). The consequences of a halt in ATP synthesis in the cell are obvious.

Peptide antibiotics that create membrane channels in the bacterial cell membrane include gramicidin and alamethicin (Bakker, 1979). Formation of membrane channels allows for passive diffusion of all ions across the cell membrane. In other words, membrane channels, unlike mobile ion carriers, are not specific to any one ion. Passive diffusion of ions through the cell membrane collapses the electrochemical potential within the cell and disrupts ATP synthesis.

Polymyxins are a group of closely related antibiotics that demonstrate activity mainly against Gram negative organisms (Korzybski et al., 1978, pg. 1572). Their mechanism of action is a result of an electrostatic interaction of the cationic antibiotic molecules with the anionic cell envelope and cytoplasmic membrane phospholipids (Kucers and Bennett, 1987, pg. 899). They also interact electrostatically with the outer membrane by competitively displacing divalent cations from the negatively charged phosphate groups of membrane lipids (Dixon and Chopra, 1986). This type of interaction creates a marked degradation of the lipid bilayer and leads to a general breakdown in the

permeability barrier, leading to a general loss of cellular material. Loss of cellular material is not limited to ions, and may include sugars, nucleic acids, amino acids, etc.

3. Inhibition of Nucleic Acid Synthesis

Peptide antibiotics can interfere with nucleic acid synthesis in a number of different ways. The antibiotics can interfere with enzymes involved in DNA replication and transcription, or they can impair the template function of the DNA directly. Such interference can lead to a loss of DNA replication and transcription which can affect all cellular processes and cause cell death.

a. Impairment of the template function of DNA

There are several interactions by which peptide antibiotics can interfere with the template function of DNA (Betina, 1983, pg. 411). The antibiotic can cross-link to the DNA strand via covalent or non-covalent interactions, it can intercalate between specific base pairs in the DNA strand, or it can degrade the DNA molecule.

Actinomycin D is classified as a chromopeptolide antibiotic (Berdy, 1974), which contains a phenoxazine chromophore joined by two pentapeptide rings (Bodanszky and Perlman, 1969) and inhibits RNA synthesis. Actinomycin intercalates between guanosine and cytosine base pairs in the DNA strand (Waring, 1970) and interferes with RNA chain elongation during transcription.

Bleomycin is a chelate forming glycopeptide (Berdy, 1974) that inhibits the synthesis of DNA. According to Umezawa (1975), the antibiotic inhibits the template function of DNA by causing single strand scissions in the DNA molecule.

b. Interference with enzymes involved in DNA replication/transcription

DNA replication and DNA transcription are very complex processes that involve many enzymes. Each step of the process and each enzyme represents a possible target for antimicrobial activity.

Edeine A is a strongly basic oligopeptide (Katz and Demain, 1977) that inhibits the replication of DNA. Edeine has little or no effect on RNA synthesis and inhibits protein synthesis at concentrations 10 fold those necessary for DNA synthesis inhibition (Kurylo-Borowska, 1975). DNA isolated from edeine-treated cells had the same melting properties and buoyant density as DNA from non treated cells. This indicates a lack of cross-linking or covalent reactions between edeine and DNA (Sadoff, 1971). Rather than binding the DNA molecule directly, edeine A appears to inhibit DNA synthesis by interfering with DNA polymerases II and III (Kurylo-Borowska, 1975). DNA polymerases are enzymes responsible for the synthesis of new DNA strands from a DNA template. A significant decrease in DNA synthesis can result in cell death.

4. Inhibitors of Protein Synthesis

Protein synthesis, like nucleic acid synthesis is a very detailed process. It involves mRNA (messenger RNA), tRNA (transfer RNA), ribosomes, and many other components

often referred to as factors. Protein synthesis can be divided into steps taking place prior to translation, and steps taking place during translation at the ribosome level (Betina, 1983, pg. 421). Prior to translation, amino acids are activated by ATP. Following activation of the amino acid by ATP, the amino acid is transferred to tRNA by a specific chemical reaction catalyzed by aminoacyl-tRNA synthetase (Brock and Madigan, 1991, pg. 164), resulting in an aminoacyl-tRNA complex that participates in protein synthesis.

Three functional phases in protein biosynthesis can be distinguished at the ribosomal level (Suzuki et al., 1989). The first step is initiation. At initiation, the ribosome recognizes the start codon on the mRNA transcript, and the ribosomal subunits (50 S and 30 S) join together with an aminoacyl-tRNA molecule and the mRNA transcript to form the initiation complex. The second step (elongation) involves a second amino acid being linked to the first one. The ribosome shifts its position on the mRNA molecule (translocation) and the elongation cycle is repeated. The third and final step is termination. Termination occurs when the ribosome complex reaches a stop codon. Subsequently the ribosome splits into its two subunits and the newly formed protein is released. Each phase has its own group of associated catalytic proteins (initiation, elongation, and termination factors) that play an integral role in the entire process. Each stage of protein synthesis represents a target for antibiotic activity.

Edeine A inhibits DNA synthesis as previously suggested, but it also interferes with protein synthesis. Edeine binds to the 30 S ribosomal subunit and prevents formation of the ribosomal initiation complex (Kurylo-Borowska, 1975), thus preventing initiation of protein synthesis. Edeine presumably inhibits binding of the ribosome to the mRNA

transcript. Other peptide antibiotics that inhibit protein synthesis include viomycin and micrococcin (Betina, 1983, pg. 428). Both of these antibiotics bind different subunits of the ribosome complex and prevent translocation of the ribosome during the elongation phase of protein synthesis. Micrococcin binds the 50 S subunit, and viomycin binds the 30 S subunit.

E. Applications of Peptide Antibiotics

1. Clinical Use of Peptide Antibiotics

a. Peptide antibiotics as antibacterial agents

The most common use of peptide antibiotics is for the treatment of infectious bacterial disease in humans. Among all of the antibacterial antibiotics in clinical use, the β-lactams (amino acid antibiotics) are probably the most important. The β-lactams are most often used to treat systemic infections; they include such antibiotics as the penicillins and the cephalosporins. β-lactam antibiotics owe their popularity to their lack of toxicity in the human body. These antibiotics act specifically against peptidoglycan synthesis which is present only in the bacterial cell wall (Brock and Madigan, 1991, pg. 364).

Use of other peptide antibiotics for clinical purposes is limited by their toxicity in the human body. Other peptide antibiotics that have been used for clinical purposes include bacitracin, gramicidin, polymyxin, and vancomycin.

Both gramicidin and bacitracin are toxic when used systemically, but have been used with much success topically. Gramicidin is often used in topical ointments to fight eye and ear infections while bacitracin has been used in conjunction with polymyxin to

provide an effective antibacterial ointment for all bacterial species (Kucers and Bennett, 1987, pg. 751). The polymyxins have been relegated to reserve drugs for fighting systemic infections because of their toxicity. Their systemic use is limited to infections caused by Gram negative bacteria that are resistant to other commonly used antibiotics (Kucers and Bennett, 1987, pg. 899). Similar to gramicidin and bacitracin, polymyxin has been used successfully to fight topical infections. Vancomycin causes several side effects including hypersensitivity, ototoxicity, and nephrotoxicity. However, it has been used successfully to fight methicillin resistant *Staphylococcus aureus* infections as well as streptococcal endocarditis in cases where the patient was allergic to the usual treatment (Kucers and Bennett, 1987, pg 1045).

b. Peptide antibiotics as antitumor agents

Peptide antibiotics have been used with some success as antitumor agents in chemotherapy. Examples of antitumor peptide antibiotics include actinomycin D and bleomycin (Berdy, 1974). Actinomycin D has been used to fight Wilms' tumor, and bleomycin has been used with some success against skin cancer. Both of these antibiotics demonstrate antitumor activity but are toxic to normal healthy cells as well, thus explaining some of the side effects of chemotherapy. They act by complexing with DNA and interfering with its template function.

2. Agricultural Applications

a. Peptide antibiotics as animal feed additives

Peptide antibiotics such as bacitracin, virginiamycin, and mikamycin have been used in low levels as additives to animal feeds (Berdy, 1974). Addition of antibiotics to animal feeds can increase feeding efficiency and lead to more rapid weight gains in livestock (Brock and Madigan, 1991, pg. 422). Rapid weight gains in livestock can shorten the time required to get an animal to market and reduce feed costs.

Addition of peptide antibiotics to animal feed has its negative aspects as well. Low level application of antibiotics to feed can lead to antibiotic resistant microflora in the gut flora of livestock which could possibly be transferred to humans. As a result, the series of antibiotics used clinically on humans differs from "feed grade" antibiotics (Berdy, 1974).

b. Peptide antibiotics as pesticides

Many of the peptide antibiotics are active against various plant pathogenic bacteria and fungi (Galvez et al., 1993). These antibiotics include such compounds as iturin, bacillomycin, mycosubtilin, and bacilysin. Gueldner et al. (1988) showed that antifungal peptides of the iturin family are active against mycelial growth of *Monilinia fructicola* which is the agent of brown rot in stone fruit. Peptide antibiotics offer a possibility for the biocontrol of plant pathogenic organisms which would decrease the use of chemical fungicides. A series of antibiotics are manufactured on a large scale for the control of plant pathogens (Berdy, 1974). Among these, blasticidin S is used very effectively in Japan for the control of *Piricularia oryzae* in rice.

c. Peptide antibiotics as food preservatives

There are a number of chemical antimicrobial agents used commercially to control microbial growth in foods. Nitrites, ethylene oxide, and propylene oxide have been shown to be detrimental to human health (Brock and Madigan, 1991, pg. 346). These compounds are still in use because the alternative is an increased risk of food poisoning. In other words, the risks involved with using these compounds are better than the alternative.

Peptide antibiotics may offer a possible alternative or adjunct to some of the chemical preservatives used in foods. The best example of a commercially successful naturally produced inhibitory agent used in foods is nisin (Jack et al., 1995). Nisin has had a long history as a food preservative with most of its applications in the dairy industry. Nisin remains the only such compound to have received GRAS (generally regarded as safe) status for use in food preservation.

Many of the bacteriocin producers have important roles in the production of fermented foods (Jack et al., 1995). Some of these organisms have been shown to be capable of inhibiting the growth of a wide variety of spoilage organisms. Some authors have proposed using bacteriocin producers in fermentation starter cultures in order to reduce the risk of starter failure due to spoilage organisms. Ruiz-Barba et al. (1994) suggested that the use of *Lactobacillus plantarum* LPC010 (a bacteriocin producer) in the starter culture for Spanish-style green olives may help control the lactic acid fermentation and reduce the risk of starter failure.

3. Other Applications

a. Applications related to the surface activity of some peptide antibiotics

Some lipopeptide antibiotics have strong surface active properties in addition to their antimicrobial properties. Surfactin, a cyclic lipopeptide produced by *Bacillus subtilis* is the most effective biosurfactant discovered so far (Cooper et al., 1981).

Microbial surface active compounds have a broad range of properties (Fiechter, 1992). They can demonstrate such properties as emulsification, phase separation, wetting, foaming, solubilization, and viscosity reduction. All of these properties make them candidates for many areas of industrial application: agriculture, food and beverage, cosmetics, pharmaceuticals, and petroleum.

Some of the most promising uses of biosurfactants are related to the petroleum industry. There is increasing interest in the use of biosurfactants in mobilizing heavy crude oil, transporting oil in pipelines, managing oil spills, oil pollution control, soil/sand remediation, and microbially enhanced oil recovery (Banat, 1995).

b. Research tools

Antibiotics play an important role in the development of biochemistry and microbiology (Berdy, 1974). Antibiotics are often used in the production of biological culture media for selective isolation of microorganisms. Antibiotics are also used as specific inhibitors of biochemical processes such that the process can be further studied and understood. Examples include inhibitors of protein synthesis, RNA/DNA synthesis, electron transport, and cation transport through cell membranes.

III. Materials and Methods

A. Organisms and Media

The antibiotic producing organism (EN 63-1) was isolated from the internal tissue of Golden Delicious apples taken from cold storage in Oliver, B.C. (Sholberg et al., 1995). EN 63-1 was grown by transferring a loopful of frozen stock to 20 ml of tryptic soy broth (Difco Laboratories, Detroit, Michigan) in a cotton plugged 150 ml Erlenmeyer flask and incubating overnight at 150 rpm and 30°C. Frozen stocks were produced by aseptically mixing 0.8 ml of an overnight culture of EN 63-1 with 0.2 ml of sterile glycerol (J.T. Baker Chemical Co., Phillipsburg, NJ) and freezing at -70°C.

The antibiotic indicator organism was *Agrobacterium vitis* CG 1005. It was grown overnight in a 150 ml Erlenmeyer flask containing 20 ml of nutrient broth (Difco) seeded with a loopful of frozen stock and incubated at 27°C and 150 rpm. Frozen stocks were created in the same manner described for the producer strain.

Other bacteria included *Staphylococcus aureus*, *Escherichia coli* (0157:H7), *Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus cereus*, *Erwinia amylovera*, *Pseudomonas corrugata*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. All of these organisms were grown by transferring a loopful of frozen stock to 20 ml of tryptic soy broth (Difco) in a 150 ml Erlenmeyer flask and incubating overnight. *Escherichia coli*, *Salmonella typhimurium*, *Bacillus cereus*, *Listeria monocytogenes*, and *Staphylococcus aureus* were all incubated at 30°C and 150 rpm, while *Erwinia amylovera*, *Pseudomonas corrugata*, *Pseudomonas fluorescens*, and *Pseudomonas putida* were incubated at 25°C

and 150 rpm. Frozen stocks were made by mixing 0.8 ml of an overnight culture with 0.2 ml of sterile glycerol (Baker) and freezing at -70°C.

Fungal strains included *Penicillium expansum* and *Botrytis cinerea*. Both fungal strains were transferred from frozen stock to potato dextrose agar (Difco) and incubated at 25°C. Frozen stocks were made by mixing 0.8 ml of a spore suspension in sterile distilled water with 0.2 ml of sterile glycerol (Baker) and freezing at -70°C. Spore suspensions were created by transferring spores from a week old fungal culture to sterile distilled water with a sterile cotton swab.

All organisms were incubated in a Lab-Line environmental shaker (Lab-Line Instruments Inc., Melrose Park, Illinois).

B. Identification of Producer Strain

1. Identification Using the Biolog Microstation System

EN 63-1 was Gram stained using the potassium hydroxide test (Suslow et al., 1982). One drop of a bacterial suspension was added to a drop of 3% (w/v) KOH (Aldrich Chemical Co., St. Louis, MO) and mixed with an inoculation loop. Gram negative organisms become gummy upon mixing, while Gram positive organisms do not. Once the Gram stain was determined, EN 63-1 was identified using the Biolog Microstation System (Biolog Inc., Hayward, CA). EN 63-1 was grown on Biolog universal growth medium + 1 % (w/v) glucose [BUGM + G] (Biolog) at 30°C for 24 hours. Colonies were aseptically removed from the BUGM plate with a sterile cotton swab and transferred to sterile 0.85 % (w/v) saline. In this manner the optical density of

the bacterial solution was adjusted to match the turbidity standards supplied by Biolog. Each well in a Gram positive MicroPlate™ was filled with 150 ul of cell suspension and the plate was incubated for 24 hours at 30°C. The plates were read and compared with the Biolog database to determine the identification of the organism.

2. Confirmation of Identification Using Biochemical Tests

A series of tests were completed according to Leary and Chun (1988) to confirm the identification of EN 63-1.

a. Motility, spore position, spore shape, swelling of bacillary body

A young broth culture (12 hours) of EN 63-1 in tryptic soy broth (Difco) was observed under a light microscope at full power (1000 x). The cells were observed for motility, spore position, spore shape, and swelling of the bacillary body.

b. Growth at 45°C

Nutrient broth (Difco), + 0.5 % (w/v) glucose (Difco) was inoculated with EN 63-1 from frozen stock. The culture was incubated at 45°C and 150 rpm in an environmental shaker (Lab-Line), and observed for growth over the course of 5 days.

c. Growth in 7 % (w/v) NaCl

Nutrient broth (Difco) + 0.5 % (w/v) glucose (Difco) + 7 % (w/v) NaCl (Difco)

was inoculated with EN 63-1 from frozen stock and grown at 30°C and 150 rpm in an environmental shaker (Lab-Line). The culture was observed several days for growth.

d. Growth at pH 5.7

Nutrient broth (Difco) + 0.5 % (w/v) glucose (Difco) was adjusted to pH 5.7 and inoculated with EN 63-1 taken from frozen stock. The culture was incubated at 30°C and 150 rpm in an environmental shaker (Lab-Line) and observed several days for growth.

e. Citrate utilization

Agar slants containing Simmons Citrate Agar (Difco) were stab inoculated and streaked with EN 63-1 from frozen stock. The slants were observed several days for growth. Citrate utilization was determined by a change in the medium from green to blue.

f. Anaerobic growth in glucose broth

Glucose broth (Cowan, 1974) made according to Leary and Chun (1988) in test tubes was inoculated with EN 63-1 from frozen stock, overlaid with sterile mineral oil and incubated at 30°C. It was observed for several days for bacterial growth.

g. Acid from arabinose, mannitol, xylose

OF basal medium slants (Difco) were made with either 1 % (w/v) arabinose (Difco), 1 % (w/v) mannitol (Difco), or 1 % (w/v) xylose (Difco) as the carbon source. Slants were stab inoculated with EN 63-1 from frozen stock, and incubated at 30°C. The

cultures were observed for several days for a colour change in the medium. Acid production was indicated by a change in the medium from green to yellow.

h. Starch hydrolysis

Starch agar plates made according to Leary and Chun (1988), were streaked with EN 63-1 from frozen stock and incubated for 5 days at 30°C. Lugol's iodine was made by dissolving 5.0 g of iodine (Aldrich) and 10.0 g of potassium iodide (Sigma Chemical Co., St. Louis, MO) in 100 ml of distilled water. A 1/5 th dilution was used to flood the starch agar plates. The starch agar plates were observed for clear colourless zones around the individual colonies, which is indicative of starch hydrolysis.

i. Acetoin production

Acetoin production was determined using the Voges-Proskauer reaction (Dye, 1968). Twenty milliliters of liquid yeast salts medium (Schaad and Stall, 1988) in a 150 ml Erlenmeyer flask was inoculated with EN 63-1 from frozen stock. The culture was incubated at 30°C and 150 rpm in an environmental shaker (Lab-Line) for five days. After 2 and 5 days the Voges-Proskauer test was completed using the method of Dickey and Kelman (1988). One milliliter of culture was placed in a test tube and mixed with 0.6 ml of a 5 % (w/v) solution of 1-naphthol (Sigma), in absolute ethanol (Baker). A 0.2 ml volume of 40 % (w/v) potassium hydroxide (Sigma) was added and mixed vigorously with the aid of a vortexer. The solution was observed at 30, 120, and 240 minutes for the production of a crimson colour which indicates a positive Voges-Proskauer reaction.

C. Production of Antibiotic

Production of the antibiotic was achieved in a minimal defined medium derived from McKeen et al. (1986). The media contained 20 g dextrose (Difco), 5 g DL-glutamic acid (Aldrich), 1.02 g $MgSO_4.7H_2O$ (BDH Chemicals, Toronto, Ont.), 1.0 g K_2HPO_4 (BDH), 0.5 g KCl (Sigma), and 1 ml of trace element solution per liter of distilled water. The trace element solution consisted of 0.5 g $MnSO_4.H_2O$ (BDH), 0.16 g $CuSO_4.5H_2O$ (BDH), and 0.015 g $FeSO_4.7H_2O$ (BDH) per 100 ml of distilled water.

One hundred milliliters of media in each of two 500 ml Erlenmeyer flasks was seeded with 1 ml of EN 63-1 taken from frozen stock. The flasks were incubated for 32 hours in a Lab-Line environmental shaker maintained at 150 rpm and 30°C.

D. 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 *Agrobacterium 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 # 3 cork borer, and 50 ul of the 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 strain appeared on the plate.

To determine the antibiotic titer, the sample was applied to the agar-well diffusion assay at 1, 5, 10, and 20 fold dilutions. The titer of a sample was calculated as the inverse

of the sample dilution giving an inhibition zone of 20 mm in diameter. The result was expressed as activity units per mililiter (AU/ml).

E. Purification of the Antibiotic

After 32 hours of incubation, the fermentation broth was split into six 50 ml centrifuge tubes and centrifuged at 15,000 x g for 10 minutes in a model J2-21M centrifuge (Beckman Industries Inc., Palo Alto, CA). The supernatant was carefully decanted and passed through a Falcon 0.22 um filter unit (Becton Dickinson and Co., Lincoln Park, NJ) to remove any remaining cells.

A bulk purification of the antibiotic was achieved by precipitating it from the culture supernatant according to McKeen et al. (1986). Concentrated HCl (Baker) was added to 50 ml of cell free supernatant until a pH of 2.0 was obtained. The resulting precipitate was recovered by centrifuging the mixture at 15,000 x g for 10 minutes in a model J2-21M centrifuge (Beckman). The supernatant was discarded and 5 ml of 50 mM Tris, pH 7.5 (Sigma), was added to the pellet. The pellet was dislodged from the bottom of the centrifuge tube and dissolved in the Tris buffer by physically disrupting it with the pestle of a teflon tissue homogenizer. Following adjustment of pH to 7.5 with 0.1 M HCl (Baker), the solution was filtered through Whatman # 4 fluted filter paper (Whatman Inc., Clifton, NJ). All purification steps to this point were carried out at room temperature.

Following acid precipitation, the crude extract was further purified by ion exchange chromatography. The crude extract (5.0 ml) was applied to a 1.5 by 15 cm Bio-Rad low pressure chromatography column (Bio-Rad Laboratories, Hercules, CA) filled

with DEAE-Cellulose (Whatman) equilibrated in 50 mM Tris, pH 7.5 (Sigma). A gradient of 0 to 0.4 M NaCl (Sigma) in Tris buffer (50 mM, pH 7.5) was run through the column at 0.5 ml/min over a period of 160 minutes followed by straight 0.4 M NaCl in Tris buffer (50 mM, pH 7.5) for 40 minutes. The eluent was monitored at 280 nm and fractions were collected every 5 minutes. An Econo System low pressure chromatography unit (Bio-Rad), operated in a 4°C chromatography refrigerator was used to run the above protocol. All fractions were tested for activity using the agar-well diffusion assay.

Liquid-liquid extraction, adapted from Morikawa et al. (1993), was used to recover the antibiotic from the ion exchange eluent. The extraction was carried out at room temperature. The active fractions from the ion exchange eluent (approximately 15 ml) were pooled and shaken vigorously with 5 ml of reagent grade isobutanol (Baker) in a 50 ml capped centrifuge tube. The resulting emulsion was centrifuged at 15,000 x g for 10 minutes in a model J2-21M centrifuge (Beckman) in order to separate the two phases of the extraction. The 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 at room temperature. The resulting residue was termed the purified antibiotic and served as the working form of the antibiotic for most of the analyses.

Further purification was achieved using an HPLC protocol modified from Hozono and Suzuki (1983). Approximately 1.5 mg of purified antibiotic was dissolved in 0.5 ml of acetonitrile:1 % (v/v) acetic acid (68:32 v/v). Fifty microliters was loaded onto a 5 um (4.0 x 250 mm) Super Pac cartridge system Pep-SC_2 C_{18} reversed phase column (Pharmacia, Uppsala, Sweden) equilibrated with acetonitrile:1 % (v/v) acetic acid (68:32

v/v). The antibiotic was eluted from the column with the same buffer at a flow rate of 1.0 ml/min. Acetonitrile was purchased from Baker and acetic acid was purchased from Aldrich. The eluent was monitored at 220 nm and 280 nm with a Waters 990 photodiode array detector (Waters Associates, Milford, MA) and the solvent was delivered to the column using a Waters 510 solvent delivery system. Each peak from the chromatogram was collected manually and tested for activity using the agar-well diffusion assay.

F. Characterization of Antibiotic (Chemical and Physical)

1. Enzymatic Treatments

Seven different enzymes were tested for their effect on the antibiotic based on a method described by Galvez et al. (1993). A purified sample of the antibiotic was used for the treatments. Each treatment consisted of the enzyme (1 mg/ml) + the antibiotic (200 ug/ml) in a final volume of 1 ml buffer (dependent on enzyme). Each test sample was incubated for the appropriate time and temperature (enzyme dependent). Positive controls (treatment without enzyme), and negative controls (treatment without antibiotic) were made up and run under the same conditions as the treatments. Both the treatments and controls were tested for activity using the agar-well diffusion assay. The activity of the treatment was compared to that of the positive control to determine whether the enzyme had any effect on the activity of the antibiotic. The negative control was used to determine whether the enzyme itself had any antimicrobial activity which could interfere with the assay.

The enzymes tested were lipase, papain, pepsin A, proteinase K, pronase E, protease VIII, and lysozyme. Table 3.1 outlines the buffer and incubation conditions that were used for each enzyme. All of the enzymes were obtained from Sigma, except for lipase which was purchased form Aldrich. All other reagents were purchased from Sigma.

Table 3.1: Incubation conditions for enzyme treatments

	Buffer	Time (h)	Temp. (°C)
Lipase	50 mM Tris, pH 7.7 + 20 mM $CaCl_2$	1	37
Papain	50 mM sodium citrate, pH 4.5 + 200 mM NaCl	1	25
Pepsin A	50 mM sodium citrate, pH 4.0	1	37
Proteinase K	50 mM Tris, pH 7.2	1	37
Pronase E	50 mM Tris, pH 7.2	1	37
Protease VIII	50 mM Tris, pH 8.0	1	37
Lysozyme	50 mM sodium citrate, pH 6.0 + 200 mM NaCl	18	25

2. Sensitivity to pH

Sensitivity to pH was determined according to Bhunia et al. (1988). Five 3 ml samples of antibiotic (200 ug/ml) were made by diluting 200 ul of a 3 mg/ml stock in 2.8 ml of distilled water. The control sample was adjusted to pH 7.5 with the addition of 200 ul of 2 M Tris, pH 7.5 (Sigma). The other four samples were adjusted to pH 3.0, pH 5.0, pH 9.0, and pH 11.0 using either 0.1 M HCl (Baker) or 0.1 M NaOH (Aldrich). All samples were incubated at room temperature for 1.5 hours. After incubation, 200 ul of 2 M Tris, pH 7.5 (Sigma) was added to each sample, excluding the control, such that all the

samples had the same pH. The volume of each sample was adjusted to 3.25 ml with distilled water and assayed in triplicate for activity using the agar-well diffusion assay.

3. Sensitivity to Temperature

Sensitivity of the antibiotic to temperature was determined at six different temperatures in each of three different buffers based on the method of Galvez at al. (1986). Six 200 ul samples of antibiotic (200 ug/ml) were made up in each buffer by diluting 13 ul of a 3 mg/ml stock solution in a final volume of 200 ul buffer. The three different buffers were 100 mM sodium citrate, pH 4.0 (Sigma); 100 mM sodium phosphate, pH 7.0 (Sigma); and 100 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), pH 11.0 (Aldrich). Samples were incubated for 30 minutes at 25°C, 37°C, 50°C, 65°C, 85°C, and 100°C. After incubation, each sample was assayed in triplicate for activity.

4. Absorbance Spectrum

The absorbance spectrum for the antibiotic was measured from the HPLC chromatogram using a Waters 990 photodiode array detector. This device allowed for the simultaneous measurement of absorbance at all wavelengths from 200 nm to 600 nm (3 nm intervals) for any compound eluting from the column.

5. Infrared Spectrum

The infrared spectrum of the antibiotic was measured as a potassium bromide pellet. Three hundred micrograms of purified antibiotic (working form) was mixed with 250 mg of KBr (Sigma) and ground to a fine powder with an agate mortar and pestle. The KBr mixture was pressed into a pellet (8 mm diameter) using a non evacuable Mini Press (Perkin-Elmer, Norwalk, Connecticut). The press consisted of a stainless steel barrel with a hardened and highly polished stainless steel bolt at either end. Approximately one third of the KBr mixture was placed into the barrel and pressed between the two stainless steel bolts. The bolts were removed and the press which contained the pellet was placed directly onto the spectrophotometer for reading. A Perkin-Elmer 1600 FTIR spectrophotometer was used to measure the spectrum. Four scans of the sample were taken.

6. Amino Acid Analysis

HPLC purification of the antibiotic was run five times, and each time the active peak was collected. The active fractions were pooled and dried under a stream of nitrogen gas. The residue was dissolved in acetonitrile: 1% (v/v) acetic acid (68:32 v/v) for amino acid analysis.

Ten microliters of the sample was applied to an Applied Biosystems amino acid analyzer consisting of a Model 420 A derivatizer and a Model 130 A separation system (Applied Biosystems, San Francisco, CA). The sample was hydrolyzed in 6 M HCl (Applied Biosystems) at 200°C for 24 hours in a sealed ampoule, derivatized with phenylisothiocyanate (Applied Biosystems), and dissolved in 0.029 M ammonium acetate

buffer, pH 5.0 (Applied Biosystems). Separation of the individual amino acids was achieved using a Brownlee 5 um (2.1 x 220 mm) C_{18} reversed phase column (Applied Biosystems). Buffer A (50 mM ammonium acetate, pH 5.4) and buffer B (acetonitrile:water [70:30]) were used to create a step gradient for eluting the amino acids from the column. The gradient is detailed in Table 3.2. Acetonitrile and ammonium acetate were purchased from Applied Biosystems. The eluent was monitored at 254 nm.

Table 3.2: Gradient used for the separation of derivitized amino acids

Time (min)	Buffer A (%)	Buffer B (%)
0	96	4
0.1	96	4
4	83	17
10	68	32
19	40	60

Norleucine (Applied Biosystems) was used as the internal standard for each run, and the device was calibrated using a PTH-Amino Acid Standard kit (Pierce Chemical Co., Rockford, IL).

7. Molecular Weight Determinations

a. SDS-PAGE

i. Gel preparation and running conditions

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 (4 % T, 3 % C), a 1.5 cm spacer gel (10 % T, 3 % C), and a 3.0 cm separating gel (16.5 % T, 3 % C). T denotes the total percentage concentration of both acrylamide monomers (acrylamide, bisacrylamide), while C denotes the percentage concentration of crosslinker relative to T (Schagger and von Jagow, 1987). Each portion of the gel was made according to Table 3.3. Acrylamide, bisacrylamide, and ammonium persulphate were purchased from Bio-Rad; Tris, TEMED, and SDS were obtained from Sigma and glycerol was purchased from Baker. The spacer gel was poured immediately following the separating gel. These two portions were allowed to polymerize prior to pouring the stacking gel.

All samples were dissolved in loading buffer and heated to 85°C for 5 minutes prior to loading the gel. Gels were run at 200 V until the dye reached the bottom of the gel (approximately 40 minutes). The recipes for the loading buffer, anode buffer, and cathode buffer are listed in Table 3.4. All reagents used to make the loading buffer, anode buffer, and cathode buffer were obtained from Sigma except for glycerol (Baker), and tricine (Aldrich).

Table 3.3: Composition of separating gel, spacer gel, and stacking gel

	Separating Gel	Spacer Gel	Stacking Gel
30 % (w/v) acrylamide stock	2.8 ml	1.65 ml	195 ul
0.5 M Tris, pH 6.8	/	/	375 ul
10 % (w/v) SDS	/	/	15 ul
[@]Gel buffer	1.65 ml	1.65 ml	/
Glycerol	0.17 g	/	/
10 % (w/v) ammonium persulphate	15 ul	15 ul	7.5 ul
TEMED	2 ul	2 ul	2 ul
Water to a final volume of	5 ml	5 ml	1.5 ml

[@]Gel buffer: 3M Tris, pH 8.45, + 0.3% (w/v) SDS

Table 3.4: Composition of loading buffer, anode buffer, and cathode buffer

	Loading Buffer	Anode Buffer	Cathode Buffer
0.5 M Tris, pH 6.8	1.0 ml	/	/
Glycerol	0.8 ml	/	/
10 % (w/v) SDS	1.6 ml	/	5.0 ml
2-β mercaptoethanol	0.4 ml	/	/
0.05 % (w/v) bromophenol blue	0.2 ml	/	/
Tris	/	12.1 g	6.06 g
Tricine	/	/	8.96 g
Adjust pH to	/	8.8	8.25
Water to a final volume of	8.0 ml	500 ml	500 ml

b. Gel staining techniques

All gels were visualized by staining with silver according to Garfin (1990). Gels were fixed for 10 minutes in 50 % (v/v) methanol (Baker), 12 % (v/v) acetic acid (Aldrich) and then washed 3 x 5 minutes in 10 % (v/v) ethanol (Baker), 5 % (v/v) acetic acid (Aldrich). The fixed gel was immersed for 5 minutes in 0.1 % (w/v) $K_2Cr_2O_7$ (Sigma) + 0.2 % nitric acid (Aldrich) and then washed with distilled water until the yellow colour

disappeared. The gel was then soaked in 0.2 % (w/v) $AgNO_3$ (Sigma) with agitation under ultraviolet light for 20 minutes, washed for 2 x 1 minute with distilled water, and finally developed with 3 % (w/v) Na_2CO_3 + 1.85 % (w/v) formaldehyde (Aldrich). The development was stopped with 1 % (v/v) acetic acid (Aldrich).

The antibiotic was located on the gel using a direct activity assay (Bhunia et al., 1987). The portion of the gel to be assayed for activity was cut out and fixed separately from the rest of the gel in 30 % (v/v) isopropanol (Baker), 10 % (v/v) acetic acid (Aldrich) for 1 hour. The fixed gel was washed for 1 hour in distilled water (several changes) to remove any SDS, and placed in a 100 by 15 mm sterile petri plate. Twenty milliliters of potato dextrose broth (Difco) + 0.75 % (w/v) agar (Difco), inoculated with 1 ml of an overnight culture of *Agrobacterium vitis*, was used to overlay the gel. The plate was incubated at 28°C until a lawn of bacteria appeared. The position of the antibiotic was indicated by a zone of inhibition in the bacterial lawn.

b. Gel filtration chromatography

Upon reviewing the methods of Galvez et al. (1993), Muriana and Klaenhammer (1991), and Upreti and Hinsdill (1973), a method was devised for gel filtration of the antibiotic. Approximately 1.5 mg of purified antibiotic (working form) was dissolved in 1 ml of 50 mM Tris, pH 7.5 (Sigma). One milliliter was applied to a 1.5 x 30 cm low pressure chromatography column (Bio-Rad) filled with Bio-Gel P-10 (Bio-Rad) equilibrated in 50 mM Tris, pH 7.5 (Sigma). The sample 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. Each fraction was 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 sulphate (Sigma) respectively. Polymyxin B sulphate (Sigma), and streptomycin sulphate (Sigma) were used as molecular weight standards. All standards were made up as 1 mg/ml stocks and run under the same conditions as the sample.

G. Characterization of Antimicrobial Activity

1. Appearance of Antibiotic Over Course of Fermentation

Production of the antibiotic over the course of a typical fermentation was followed using the method of Gonzalez et al. (1994). One hundred milliliters of antibiotic production media was made up in each of three 500 ml Erlenmeyer flasks, and inoculated with 1 ml of EN 63-1 taken from frozen stock. All three flasks were incubated at 30°C and 150 rpm in an environmental shaker (Lab-Line). Each flask was monitored over a different 12 hour period for antibiotic activity and optical density ($OD_{600\ nm}$) to determine at which stage of the fermentation the antibiotic is produced. The last flask was monitored for 14 hours and the fermentation was monitored for a total of 38 hours.

Optical density (600 nm) was measured with a Lambda 3B UV/vis spectrophotometer (Perkin Elmer) using a quartz cuvette with a 1 cm light path. Samples giving an optical density greater than 0.7 were diluted such that the OD reading was

between 0.1 and 0.7. The actual optical density was obtained by multiplying the optical density reading by the dilution factor.

The antibiotic activity of the fermentation broth was determined by applying a cell free sample of the culture supernatant to the agar-well diffusion assay. One milliliter of the fermentation broth was centrifuged at 16,000 x g for 5 minutes in an Eppendorf model 5415 C microcentrifuge (Brinkman Instruments Inc., Westbury, NY). The supernatant was carefully removed from the cell mass and passed through a 0.45 um sterile syringe filter (Gelman Sciences, An Arbor, MI) to remove any remaining cells. Fifty microliters of the cell free supernatant was assayed for activity.

2. Activity Spectrum

Twelve different organisms were tested for their sensitivity to the antibiotic using the agar-well diffusion assay. Aliquots of an overnight culture of the bacteria being assayed for sensitivity to the antibiotic were added to sterile 1 % (w/v) peptone (Difco) 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 *Agrobacterium 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, West Germany). The organisms tested and their growth conditions for the assay are outlined in Table 3.5.

3. Effect of Antibiotic on *Agrobacterium vitis*

The effect of the antibiotic on *Agrobacterium vitis* was studied according to the methods of Gonzalez et al. (1994), Galvez et al. (1986), and Meyers et al. (1973). *Agrobacterium vitis* was grown overnight in 20 ml nutrient broth (Difco) in a 150 ml Erlenmeyer flask. Five milliliters of the overnight culture was used to seed each of three 30 ml volumes of nutrient broth (Difco) in a 250 ml Erlenmeyer flask. All three flasks were incubated in an environmental shaker (Lab-Line) at 28°C and 150 rpm for 4 hours. At 4 hours, purified antibiotic (working form) was added to flask #2 (10 ug/ml) and flask #3 (50 ug/ml). Flask #1 (control) had no antibiotic added to it. Each flask was assayed over the course of several hours for optical density (600 nm) and viable cells (CFU/ml).

Table 3.5: Organisms and growth conditions for activity spectrum assay

	Media	Temperature (°C)
Escherichia coli	tryptic soy agar	30
Salmonella typhimurium	tryptic soy agar	30
Erwinia amylovera	tryptic soy agar	25
Agrobacterium vitis	potato dextrose agar	25
Pseudomonas putida	tryptic soy agar	25
Pseudomonas fluorescens	tryptic soy agar	25
Pseudomonas corrugata	tryptic soy agar	25
Listeria monocytogenes	tryptic soy agar	30
Bacillus cereus	tryptic soy agar	30
Staphylococcus aureus	tryptic soy agar	30
Penicillium expansum	potato dextrose agar	25
Botrytis cinerea	potato dextrose agar	25

Optical density measurements were made with a Lambda 3B UV/vis spectrophotometer (Perkin Elmer) using a quartz cuvette with a 1 cm light path. Viable cell counts were obtained using a dilution plating scheme. Culture samples were diluted (10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7}) and 100 uL was spread plated on a 100 x 15 mm petri plate containing nutrient agar (Difco). The plates were incubated at 28°C until viable counts could be made.

IV. Results and Discussion

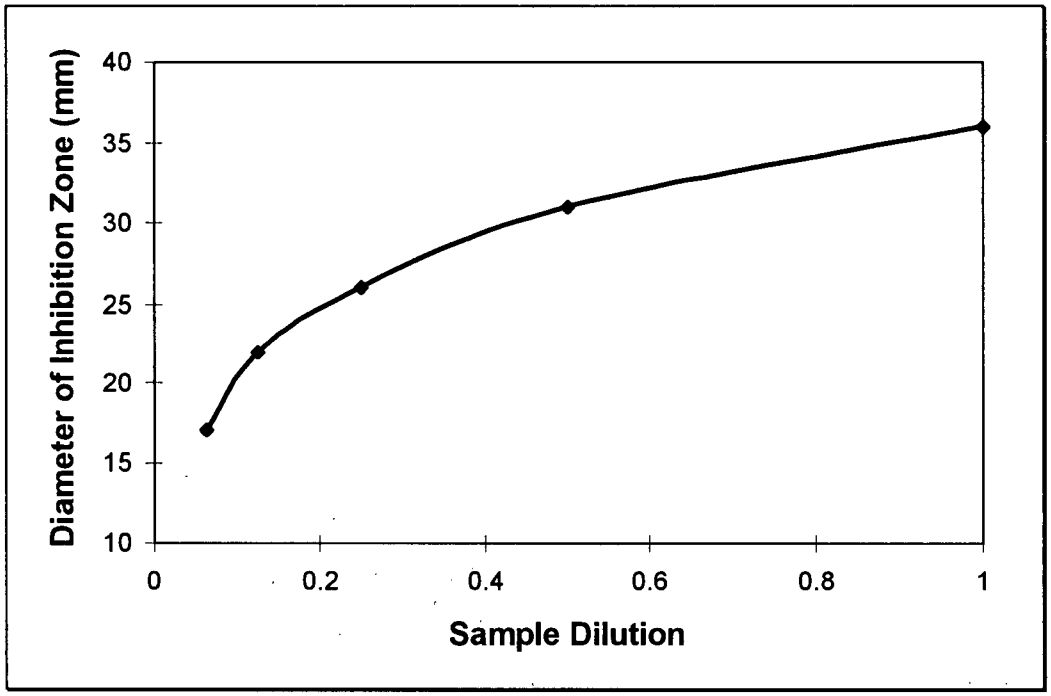
A. Identification of Antibiotic Producing Organism

The antibiotic producing organism (EN 63-1) was Gram stained and determined to be a Gram positive rod. Testing with the Biolog Microstation System gave a positive identification of EN 63-1 as *Bacillus brevis* (similarity index = 0.787). Upon repeating the identification, the Biolog system gave a positive identification of EN 63-1 as *Bacillus subtilis* (similarity index = 0.623). From these results, EN 63-1 was determined to be a species of *Bacillus*. Although these results were not conclusive (i.e. no species identification was made) it was a quick and effective means for determining the genera of EN 63-1. It is far easier to determine the proper microbiological tests for bacterial identification if the genera of the organism is already known.

The organism was further identified to the species level (Table 4.1) using a series of microbiological tests described by Leary and Chun (1988). EN 63-1 showed no bacillary swelling or anaerobic growth in glucose broth, but produced positive results for all other tests. According to Leary and Chun (1988), *Bacillus subtilis* gave similar results; however, *Bacillus brevis* was quite different (see Table 4.1). On the basis of these findings, EN 63-1 was identified as *Bacillus subtilis*. This confirms the genera determination by the Biolog system and identifies the organism to the species level.

B. 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



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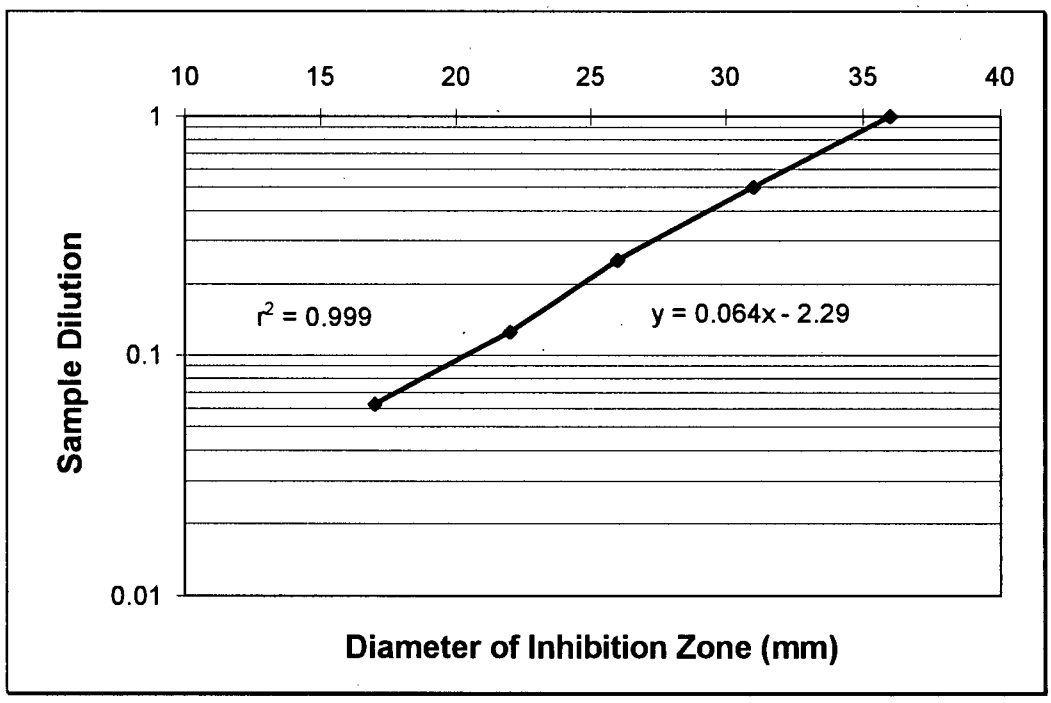


B

Figure 4.1: Dose response curves for the antibiotic. Relationship between zone of inhibition and antibiotic concentration (A). Relationship between log of of antibiotic concentration and zone of inhibition (B).

Table 4.1: Identification of EN 63-1 to the species level

Test	@Bacillus brevis	@Bacillus subtilis	EN 63-1
Gram reaction	/	+	+
Motility	+	+	+
Oval spores	+	+	+
Central spore	+	+	+
Bacillary swelling	+	-	-
Growth at 45°C	+	+	+
Growth at pH 5.7	+	+	+
Growth in 7% (w/v) NaCl	variable	+	+
Citrate Utilization	variable	+	+
Anaerobic growth in glucose broth	-	-	-
Acid from arabinose	-	+	+
Acid from mannitol	variable	+	+
Acid from xylose	variable	+	+
Voges-Proskauer test	-	+	+
Starch hydrolysis	-	+	+

@ Results obtained from Leary and Chun (1988)

diffusion assay. If the zone of inhibition is plotted against sample dilution, a linear relationship is not observed (Figure 4.1 A). The dose-response curve tends to flatten out as the dilution factor decreases. In other words, as the sample concentration increases, the slope of the curve decreases.

According to Betina (1983, pg. 105) a linear response is observed if the log of the antibiotic concentration is plotted against the zone of inhibition. Figure 4.1 B (log of the sample dilution versus the inhibition zone) demonstrates this relationship. Regression analysis of the data showed a very strong linear relationship ($r^2 = 0.999$). Such a high correlation coefficient suggests that there is little variability in the agar diffusion assay within the range tested.

This relationship can be used to calculate the dilution factor for a given sample that would yield a particular zone of inhibition. In other words, if the log of several sample dilutions is plotted against the corresponding inhibition zones, the equation for the resulting line can be used to calculate the sample dilution that would give a particular zone of inhibition. This reduces the number of dilutions that need to be made for each sample in order to calculate the sample activity, and the result is quite accurate based on the strength of the linear relationship.

C. Purification of Antibiotic

The effectiveness of each step of the purification protocol was determined using the specific activity ($AU/ml/A_{280}$) of the antimicrobial preparation at each stage of the purification protocol (Table 4.2), according to the method of Holo et al. (1991). Specific activity was calculated by dividing activity (activity units/ml) by absorbance (A_{280}).

1. HCl Precipitate of Culture Supernatant

The cell free culture supernatant served as the starting point for the purification of the antibiotic. Bulk purification of the antibiotic was achieved by decreasing the pH of the culture supernatant to 2.0. The antibiotic came out of solution as a rusty brown precipitate which could easily be removed from the supernatant by centrifugation. The resulting pellet dissolved slowly upon addition of 5 ml of 50 mM Tris, pH 7.5.

Based on the recovery of activity (Table 4.2), the bulk purification step recovered 103 % of the activity from the culture supernatant, and increased the specific activity 3.3

Table 4.2: Purification of antibiotic from a 32 hour old culture supernatant of *Bacillus subtilis.*

Sample	Volume (ml)	Activity (AU/ml)	Absorbance (A_{280nm})	Specific Activity ($AU/ml/A_{280}$)	*Yield (%)	Fold Purification
Culture spnt.	50	72	5.9	12	/	/
HCl precipitate	5.0	743	19	39	103	3.3
Ion exchange peak	15	56	2.2	25	23	2.2
Butanol extract	0.5	2416	26	93	34	7.8
HPLC peak	0.5	1320	14	94	18	8.1

Note: Buffer conditions and purity were different at each stage of the purification protocol. It is difficult to determine how this may have affected activity calculations.
*Yield was calculated as % of total activity (AU/ml x Sample volume) recovered.

fold. It is important to keep in mind that the activity recovered is not necessarily a good indication of antibiotic recovery, because the activity of the antibiotic may be affected by buffer conditions and purity. The buffer conditions and purity were different at each stage of the purification protocol. 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, while Smidt and Vidaver (1986) reported a 115 % recovery in activity of syringacin W-1 upon concentration of the ultrafiltration retentate.

2. Ion Exchange Purification

Four peaks were obtained by ion exchange chromatography of the acid precipitate (Figure 4.2). Antibiotic activity corresponded with the fourth and largest peak on the chromatogram. The active peak was not collected from baseline to baseline because of significant peak overlap. To minimize the contamination of the antibiotic with the overlapping peaks, a substantial portion of the active peak was not collected (Figure 4.2).

Only 23 % of the initial activity was recovered by ion exchange chromatography and the specific activity actually decreased from 39 for the previous purification step to 26. A decrease in the specific activity is best explained by a loss of activity in the antibiotic. When the antibiotic was left in the ion exchange eluent overnight, a complete loss of activity was observed (data not shown). The apparent loss of activity in the antibiotic, and the fact that only a portion of the active peak was collected explain the poor recovery of activity. These results are not surprising. A poor recovery of antibiotic activity after ion exchange chromatography was observed by Gonzalez et al. (1994), and Smidt and Vidaver (1986).

At the expense of decreasing the activity of the antibiotic, the ion exchange protocol was very effective at removing the colored material contaminating the sample. Most of the rusty brown material contaminating the antibiotic was bound very strongly to the column and could not be removed with either dilute NaOH or 2.0 M NaCl. As well, the ion exchange step separated the antibiotic from the other three peaks in the chromatogram.

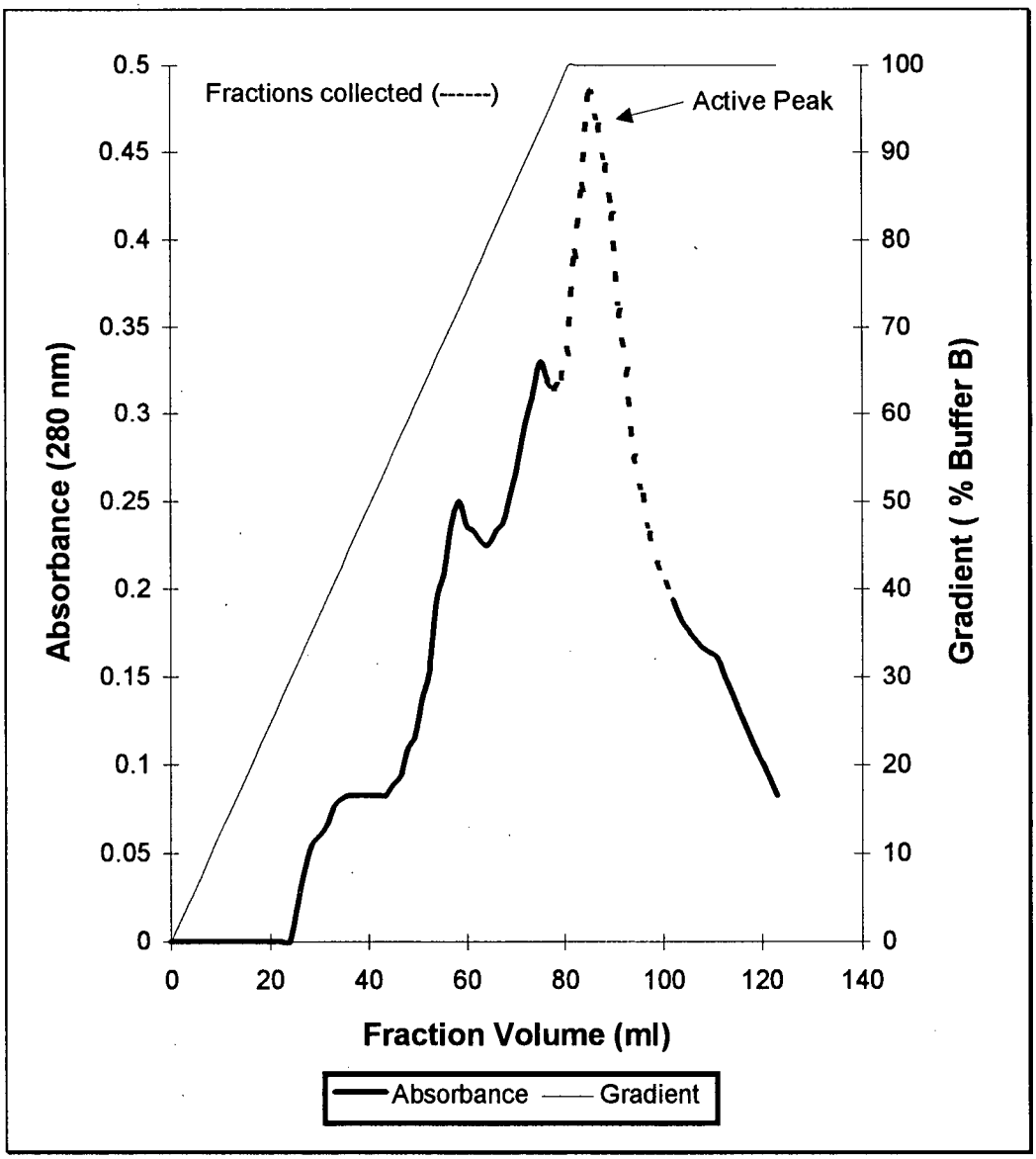


Figure 4.2: Elution pattern of the HCl precipitate from DEAE-Sephacel equilibrated in 50 mM Tris buffer, pH 7.5 (Buffer A). The antibiotic was eluted from the column with a gradient of 0 to 100 % Buffer B (50 mM Tris, pH 7.5, containing 0.4 M NaCl). Flow: 0.5 ml/min.

3. Butanol Extract of Ion Exchange Peak

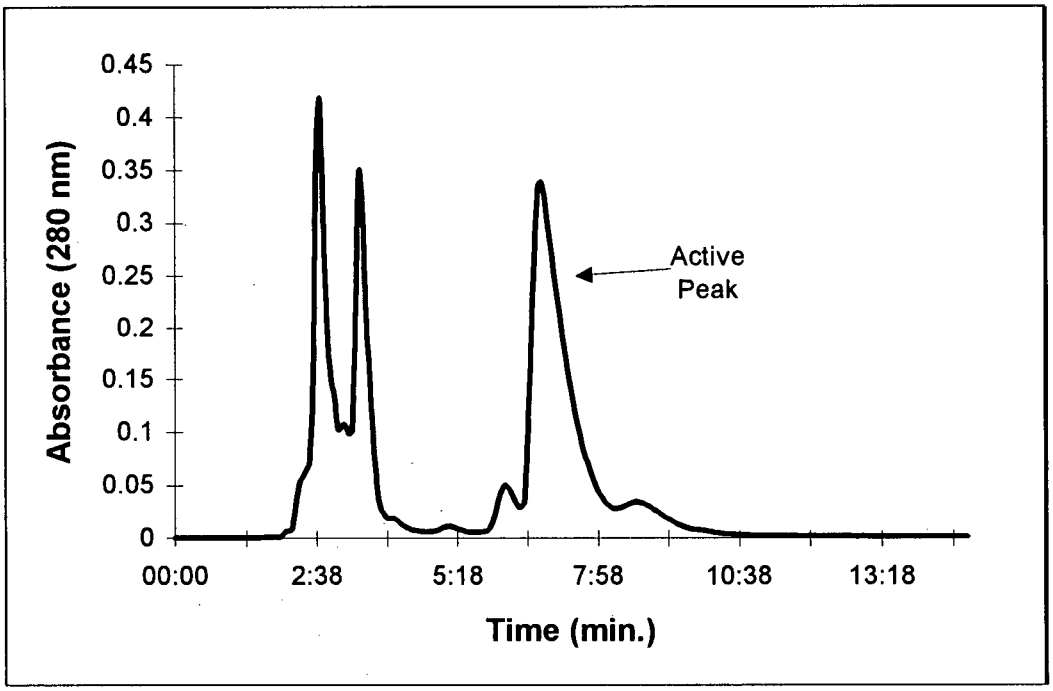
The ion exchange sample was desalted and concentrated by extraction with isobutanol. Upon drying the butanol extract, the resulting antibiotic preparation was present as an amorphous pale yellow powder that stuck to the sides of the glass centrifuge tube in which it was dried. At this stage the antibiotic was readily soluble in distilled water, 95 % (v/v) ethanol, acetonitrile:1 % (v/v) acetic acid (68:32 v/v), methanol, and 50 mM Tris (pH 7.5).

Butanol extraction afforded a 34 % recovery of the initial activity and increased the initial specific activity 7.8 fold. The butanol extract served as the working form of the antibiotic for all other analyses excluding the amino acid analysis.

4. HPLC Purification

HPLC purification of the antibiotic served two purposes. It provided a purified sample of the antibiotic for amino acid analysis, and it determined the overall purity of the sample. Based on the HPLC chromatogram (Figure 4.3 A), it appears as though there was a fair amount of contamination in the antibiotic preparation. A significant amount of ultraviolet absorbing material was not retained by the HPLC column and eluted in the early stages of the purification.

To determine whether this material was a derivative of the antibiotic or actual contamination, the active peak (Figure 4.3 A) from three HPLC runs was pooled, concentrated, redissolved in loading buffer, and reinjected onto the HPLC column. The resulting chromatogram (Figure 4.3 B) was remarkably similar to the original



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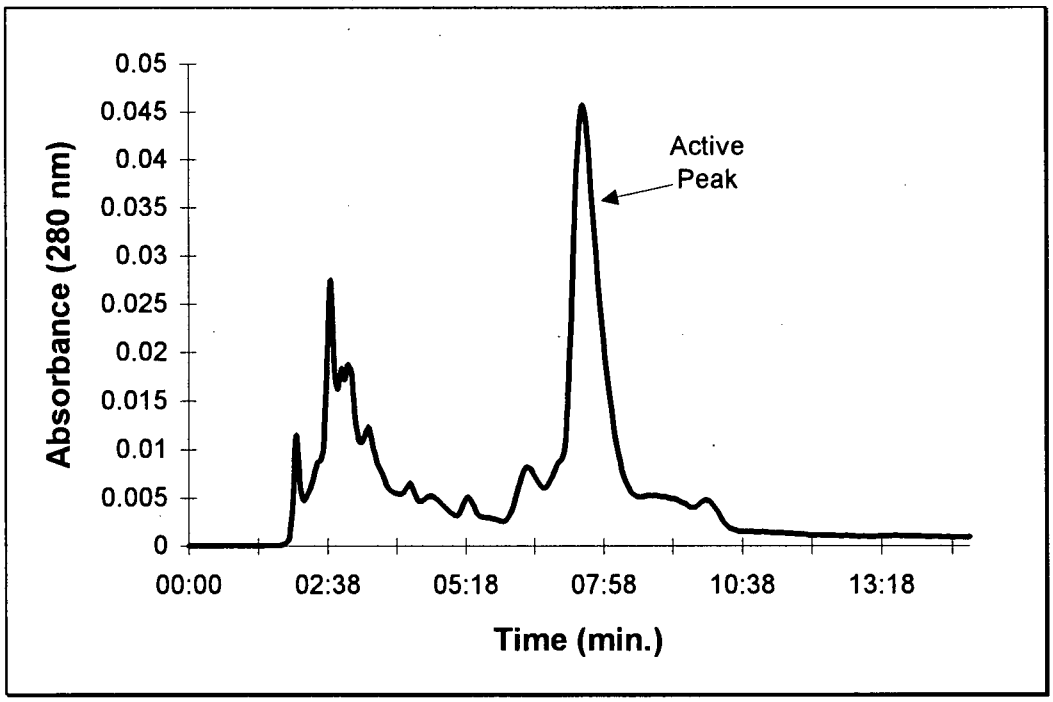


B

Figure 4.3: Elution pattern of the antibiotic (butanol extract of ion exchange peak) on a reversed-phase C_{18} column. (A). The active peak from the initial HPLC run was collected, concentrated and run on the HPLC column again (B). Flow rate - 1 ml/min. Eluent - acetonitrile: 1 % (v/v) acetic acid (68:32 v/v).

chromatogram. Interestingly, the absorbance spectrum (Figure 4.4 A) of the active peak collected for repurification differed markedly from the absorbance spectrum (Figure 4.4 B) of the material eluting in the early stages of the repurification. Apparently, the material that elutes in the early stages of the HPLC run is a derivative of the antibiotic.

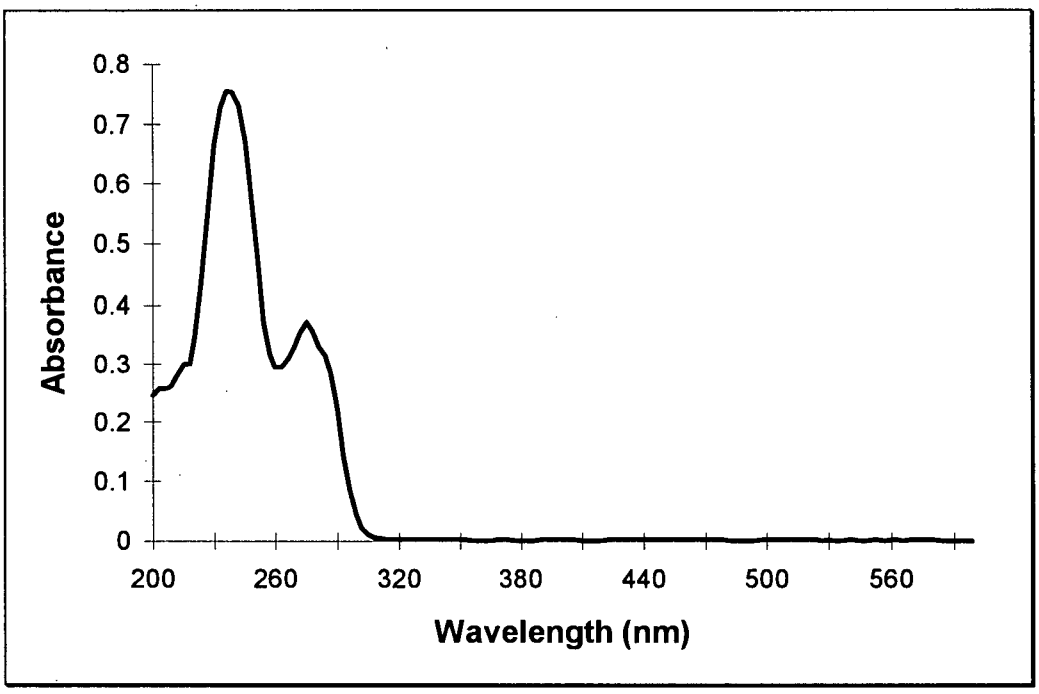
If the apparent derivatized material is disregarded, it looks as though the antibiotic preparation is quite pure. Two other peaks were present in the chromatogram, eluting just prior to and just after the active antibiotic. Both of these peaks were relatively minor and the peak resolution was satisfactory.

HPLC purification of the antibiotic yielded an 18 % recovery of the initial activity and increased the initial specific activity 8.1 fold (Table 4.2). It is important to mention that the antibiotic was no longer soluble in distilled water or 50 mm Tris buffer (pH 7.5) after HPLC purification. It had to be dissolved in 95 % (v/v) ethanol for total activity and absorbance calculations. It is difficult to determine how this may have affected the activity and absorbance calculations.

D. Physical and Chemical Characterization of the Antibiotic

1. Sensitivity to enzyme hydrolysis

Table 4.3 demonstrates the effect of seven different enzymes on the activity of the antibiotic. None of the enzymes tested caused a significant ($p < 0.05$ for a paired t-test) reduction in the zone of inhibition of the treatment sample. This suggests that none of the enzymes had any effect on the integrity of the antibiotic.



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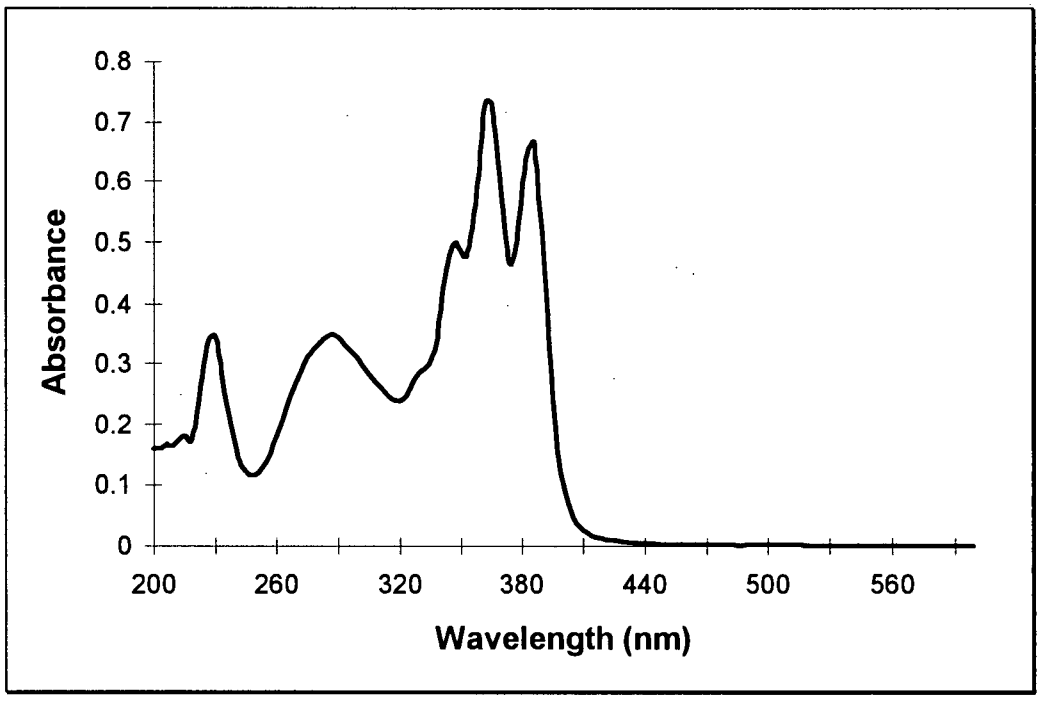


B

Figure 4.4: UV/visible absorbance spectrum of active peak seen in Figure 4.3 A, the initial HPLC chromatogram (A). UV/visible absorbance spectrum of contaminating peaks (retention time = 3.5 minutes, Figure 4.3 B) from repurification of the active peak (B).

According to Katz and Demain (1977), it is common for peptide antibiotics to be resistant to hydrolysis by peptidases and proteases of plant and animal origin. This is not surprising because peptide antibiotics often contain less than 15 amino acids in their structure, many of which are unusual and not found in proteins; thus hydrolysis sites for the hydrolytic enzymes are not present. The larger peptide antibiotics (bacteriocins), which contain more amino acids, provide

Table 4.3: Effect of hydrolytic enzymes on antibiotic

Enzyme	Mean Zone of Inhibition (mm)		@Significance (p)
	Treatment	Control	
Lipase	26 +/- 1	26 +/- 0	.21
Papain	29 +/- 1	29 +/- 1	.5
Pepsin A	28 +/- 0	29 +/- 1	.092
Proteinase K	28 +/- 1	28 +/- 1	.11
Pronase E	26 +/- 1	27 +/- 1	.37
Protease VIII	27 +/- 0	27 +/- 0	.21
Lysozyme	23 +/- 0	23 +/- 1	.21

@Level of significance for a paired t-test between treatment and control. Treatment and control samples were assayed in triplicate for inhibition zones

more possible hydrolysis sites and are usually more sensitive to proteolytic attack. As well, the "bacteriocins" usually do not contain as many unusual amino acids in their structure.

2. Sensitivity to pH

Figure 4.5 demonstrates the effect of pH on the stability of the antibiotic, which was determined by measuring the zone of inhibition of the sample after pH treatment. It is

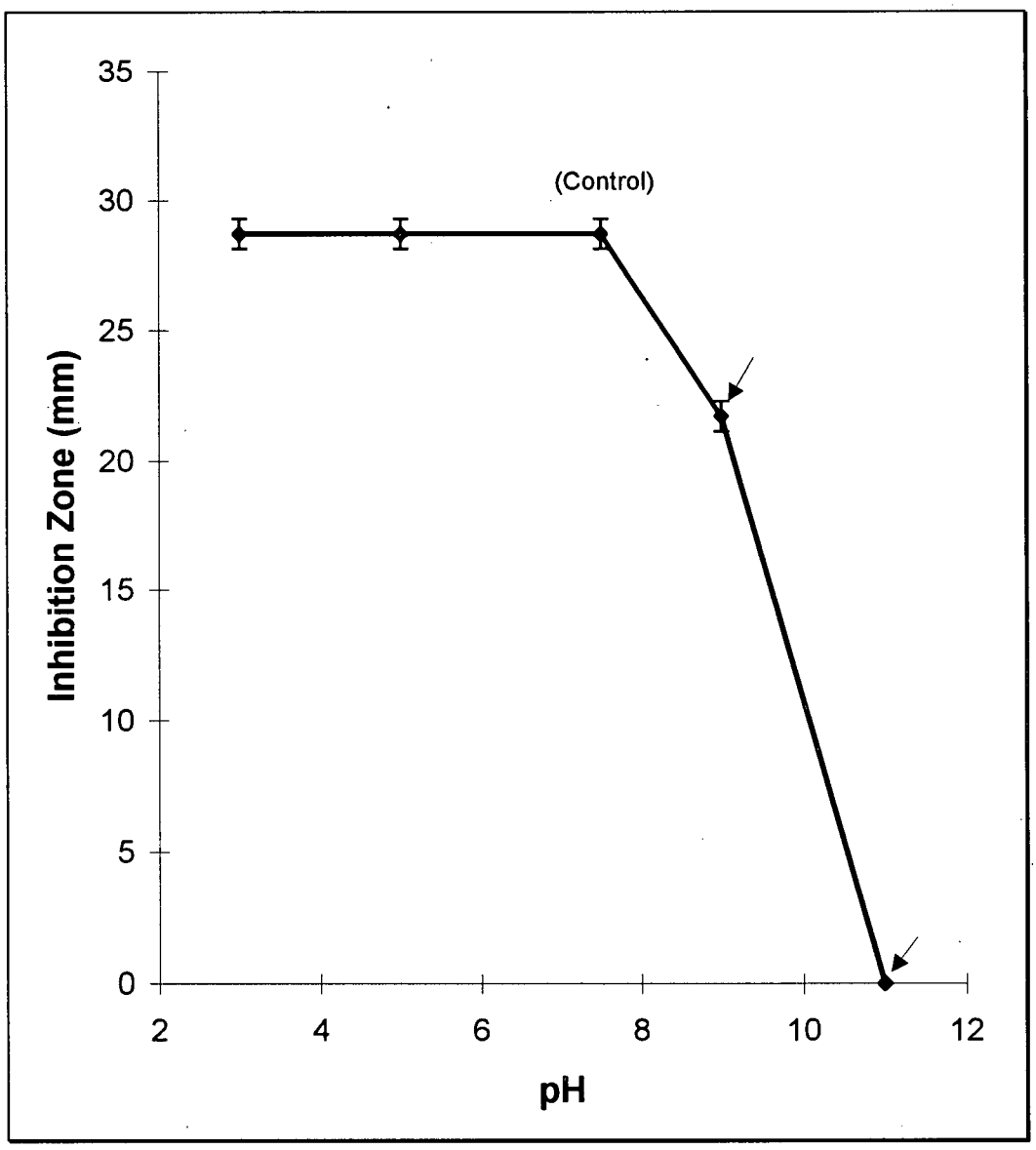


Figure 4.5: Effect of pH on the activity of the antibiotic. Points marked with an arrrowhead are significantly different from the control ($p<0.05$ for a paired t test). All samples were assayed in triplicate for zone of inhibition. Error bars represent standard deviation.

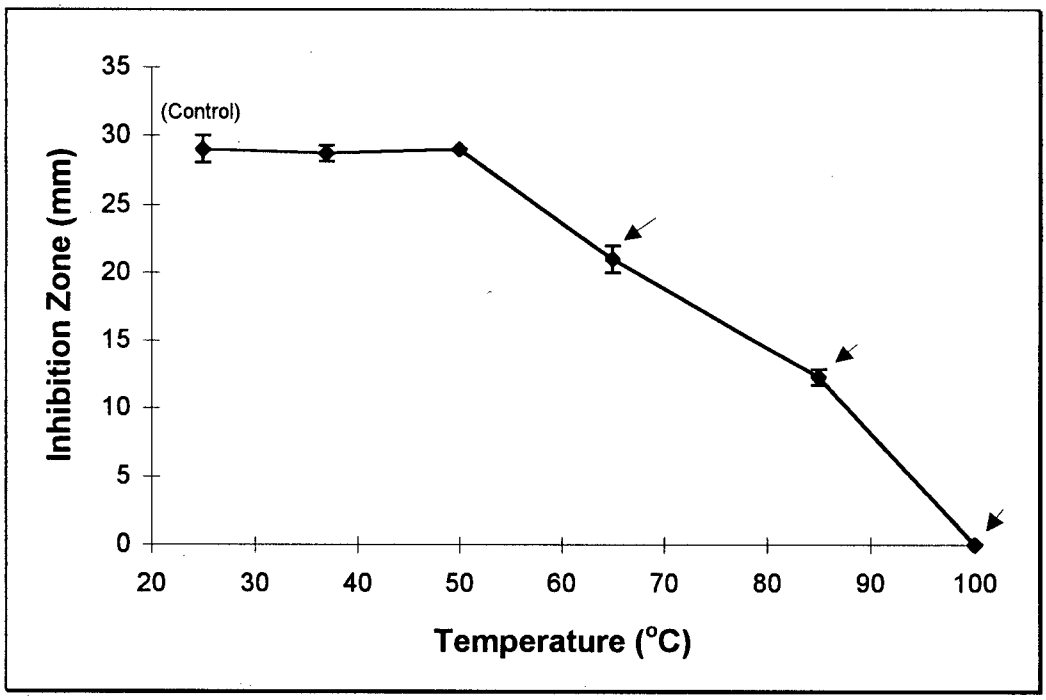
important to note that all samples were adjusted to pH 7.5 with Tris buffer prior to assaying them for activity.

The pH 9.0 and pH 11.0 samples are the only two treatments that showed a significantly decreased ($p < 0.05$ for a paired t-test) zone of inhibition relative to the control (pH 7.5). The antibiotic appears to be stable up to a pH value of approximately 8.0. Beyond this pH the antibiotic begins to lose activity. It was completely inactivated above a pH of approximately 10. Many antibiotics demonstrate a similar sensitivity to high pH. A peptide antibiotic (AS-48) produced by *Streptococcus faecalis* (Galvez et al., 1986) was stable at neutral and acidic pH, but gradually became inactivated at basic pH. Another example, peptide A12-C produced by *Bacillus licheniformis* (Galvez et al., 1993), demonstrated similar sensitivity to basic pH.

In hindsight, the ion exchange chromatography protocol carried out during purification of the antibiotic, should have been carried out at pH 6.5 rather than pH 7.5 in order to increase the stability of the antibiotic. This may have increased the recovery of antibiotic activity from the ion exchange column

3. Sensitivity to temperature

The effect of temperature on the antibiotic was determined for six different temperatures at two different pH values (Figure 4.6). All samples and controls were incubated under these conditions for 30 minutes. Figure 4.6 A details the effect of temperature on the antibiotic at an acidic pH (pH 4.0). The last three data points (65°C, 85°C, 100°C) all showed a decreased zone of inhibition ($p < 0.05$ for a paired t-test) when



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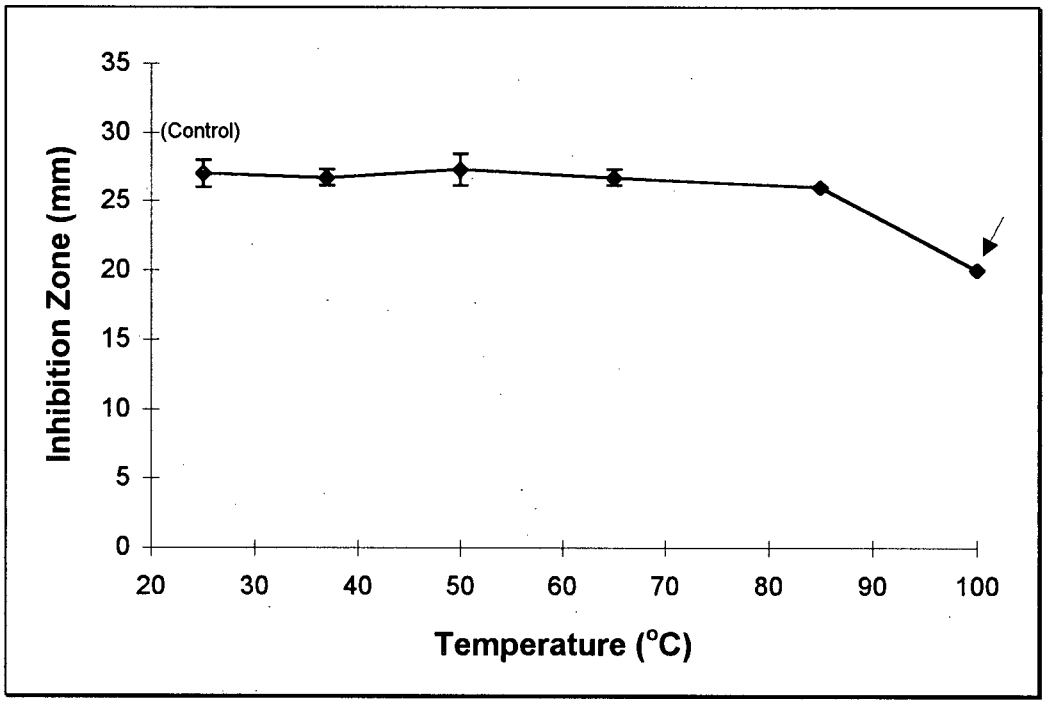


B

Figure 4.6: Sensitivity of antibiotic to temperature at pH 4.0 (A). Sensitivity of antibiotic to temperature at pH 7.0 (B). Data points marked by an arrowhead are significantly different than the control ($p<0.05$ for a paired t test). All samples were assayed in triplicate for zone of inhibition. Error bars represent standard deviation.

compared to the control (25°C). Based on these findings, it appears as though the antibiotic gradually loses activity above 50°C. A complete loss of activity was observed at 100°C.

The effect of temperature on the activity of the antibiotic at a neutral pH is seen in Figure 4.6B. Only the last data point (100°C) showed a significant decrease in activity ($p < 0.05$ for a paired t-test) when compared to the control (25°C). The antibiotic was far more resistant to increased temperature at neutral pH than at an acidic pH. At pH 7.0, the antibiotic began to lose activity only above 85°C, and a complete loss of activity at 100°C was not observed.

The antibiotic was subjected to the same experiment at pH 11.0 (data not shown), and a complete loss of activity was observed for all data points regardless of temperature. This is in agreement with the results from Figure 4.5 which suggest a complete loss of activity above a pH of 10.0 at room temperature.

Resistance to temperatures above 85°C has been reported for many antibiotics. Included are pediocin AcH (Bhunia et al., 1988), peptide A12-C (Galvez et al., 1993), and staphylococcin 1580 (Jetten and Vogels, 1972) to name a few.

4. Ultraviolet spectrum

The absorbance spectrum for the antibiotic was measured between 200 and 600 nm; however, there was no appreciable absorbance above 300 nm. Figure 4.7 gives the ultraviolet absorbance spectrum for the antibiotic between 200 and 320 nm. The absorbance pattern is not characteristic of most peptide antibiotics which usually

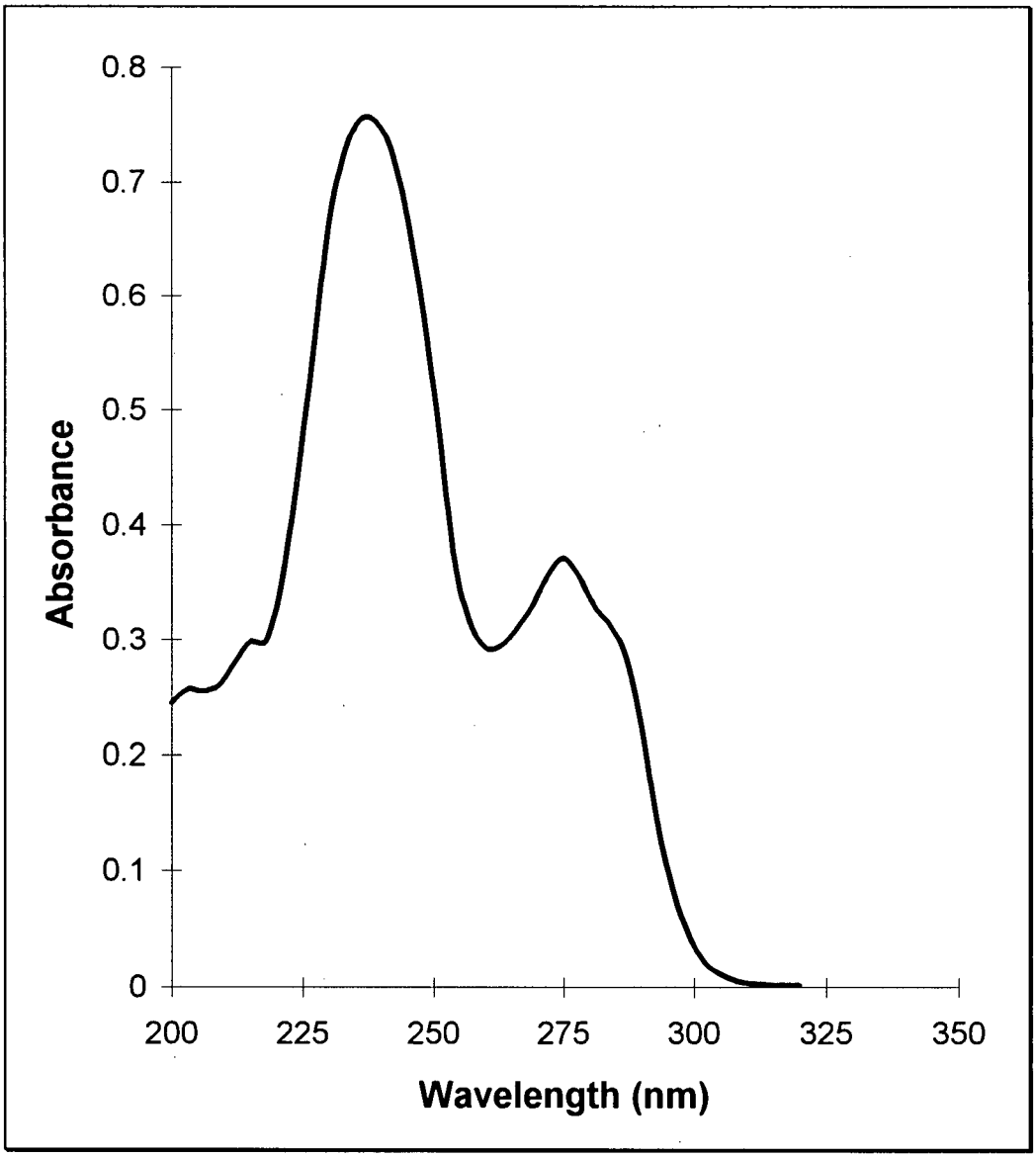


Figure 4.7: Ultraviolet absorbance spectrum of HPLC purified antibiotic in acetonitrile: 1 % (v/v) acetic acid (68:32 v/v).

demonstrate terminal absorbance patterns. Rather, the antibiotic had absorbance maxima at 235 nm, 278 nm, and 285 nm in acetonitrile: 1% (v/v) acetic acid (68:32 v/v). This is very similar to the absorbance pattern given by the peptide antibiotic LI-FO3 produced by *Bacillus polymyxa* (Kurusu and Ohba, 1987). LI-FO3 showed absorbance maxima at 224 nm, 278 nm, and 285 nm in methanol. According to Kurusu and Ohba (1987) this absorbance pattern indicates the presence of a tyrosine residue. Similar ultraviolet absorbance patterns have been reported for enduracidin (Asai et al., 1968) and janiemycin (Meyers et al., 1970). Neither of those antibiotics contained tyrosine in its structure; however, both contained α-amino-4-hydroxyphenylacetic acid which is very similar to tyrosine.

5. Infrared spectrum

The Fourier transform infrared spectrum (Figure 4.8) offers some valuable information about the antibiotic structure. Much can be inferred about the structure of the antibiotic by comparing its spectrum to those of similar antibiotics.

The FTIR spectrum offered the first concrete evidence that the antibiotic contained a peptide in its structure. Characteristic absorption valleys at 3300 cm^{-1}, 1650 cm^{-1}, and 1530 cm^{-1}, resulting from the (N-H) stretching mode, the (>C=O) stretching mode, and the deformation mode (combined with the C-N stretching mode) of the N-H bond, respectively (Jenny et al., 1991) indicated that the antibiotic contained some peptide bonds. Prior to this information, the antibiotic was only assumed to be a peptide based primarily on molecular weight and its ultraviolet spectrum. Absorbance valleys resulting

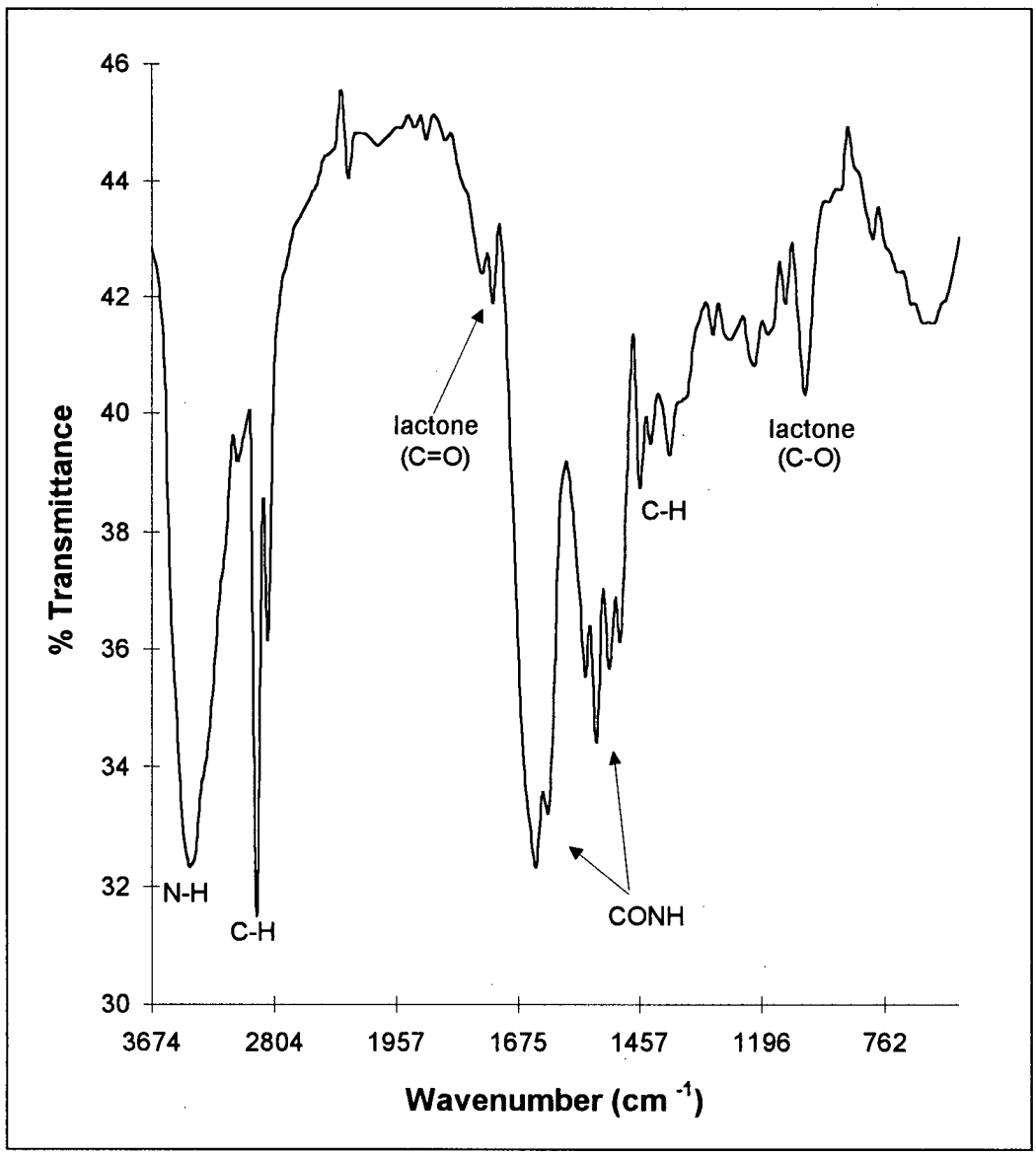


Figure 4.8: Fourier transform - IR spectrum of antibiotic (KBr pellet).

from aliphatic chains ($-CH_3$, $-CH_2$) at 2950 cm^{-1}, 2850 cm^{-1}, 1460 cm^{-1}, and 1400 cm^{-1} suggested the presence of an aliphatic chain in the antibiotic structure (Morikawa et al., 1993). It is important to note that these valleys could have been a result of the functional groups of nonpolar amino acids; however, based on the strength of the absorption at these wavenumbers, the former explanation is more likely. Finally, the small valley present at 1740 cm^{-1} suggests lactone carbonyl absorption (Jenny et al. 1991). The sharp valley at 1060 cm^{-1}, may have also resulted from a lactone bond. According to Silverstein and Bassler (1967), the C-O stretching mode of lactones give rise to bands in the 1300-1000 cm^{-1} region; however, the frequencies of these bands are generally much less reliable than the carbonyl bands.

Based on these findings, the 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 antibiotic in question appears to belong to a subclass of peptolides that contain fatty acids in their structure. The FTIR spectrum of the antibiotic 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 demonstrate strong surface active properties and for this reason are sometimes classified as biosurfactants.

Many other lipopeptides described in the literature do not contain lactone bonds in their structure. Examples include polymyxin T_1 (Shoji et al., 1977), iturin (Gueldner et al.,

1988), and cerexins C and D (Shoji et al., 1976). These peptides demonstrate a very similar infrared spectrum to the antibiotic in question; however, they do not show a valley at 1740 cm^{-1} which is characteristic of lactone carbonyl stretching.

6. Amino Acid Analysis

Table 4.4 gives the results of the amino acid analysis. Sixteen peaks were identified by the amino acid auto analyzer; however, only eight of them were above the threshold value. Based on these findings, the antibiotic appears to contain aspartic acid, glutamic acid, serine, glycine, alanine, proline, valine, and leucine (2:3:1:1:1:1:1:4). It is important to note that acid hydrolysis converts the side chain amides of asparagine and glutamine to the side chains of aspartate and glutamate (Rawn, 1989). Thus, the molar ratios of aspartic acid and glutamic acid refer to the combined totals of either asparagine/aspartate or glutamine/glutamate, respectively. Based on the acidic properties of the antibiotic (precipitates at pH 2.0) and the lack of other charged amino acids in the structure, one would assume that both of these amino acids are present predominantly in the acidic form.

Interestingly, the antibiotic does not contain an aromatic amino acid as was suggested by its ultraviolet absorbance spectrum (Figure 4.7). Tyrosine was one of the 16 identified peaks in the amino acid analysis; however, its recovery was just below the accepted threshold. It is possible that the ultraviolet absorbance pattern of the antibiotic is a result of some unusual aromatic amino acid that appeared as an artifact in the amino acid analysis. It is important to note that tryptophan (which is destroyed by acid hydrolysis) was not quantitated for by using an enzymatic hydrolysis because there was no evidence of its presence in the automated amino acid composition report.

Table 4.4: Composition report for the automated amino acid analysis.

Peak identification	Retention time (min)	Composition by residue	Number of residues
[1]**Aspartic acid**	6.33	1.85	**2**
[1]**Glutamic acid**	6.72	2.59	**3**
Serine	7.57	0.86	**1**
Glycine	7.99	1.27	**1**
Histidine	8.41	0.07	/
Arginine	9.05	0.08	/
Threonine	9.34	0.20	/
Alanine	9.68	1.00	**1**
Proline	9.98	0.66	**1**
Tyrosine	12.29	0.36	/
Valine	13.17	1.29	**1**
Methionine	13.49	0.08	/
Isoleucine	15.10	0.30	/
Leucine	15.3	3.52	**4**
Phenylalanine	15.98	0.08	/
Lysine	16.98	0.12	/

The presence of an aromatic amino acid (such as tyrosine or tryptophan) was suggested by the ultraviolet spectrum; however, this was not substantiated in the composition report for the amino acid analysis.
[1]Aspartic acid and glutamic acid refer to aspartate/asparagine and glutamate/glutamine respectively.

7. Molecular Weight Determination

a. SDS PAGE

The SDS polyacrylamide gel (Figure 4.9) offered an approximation of the molecular weight of the antibiotic. The antibiotic (Lane 3) was significantly smaller than any of the molecular weight markers run in lane one, the smallest of which was α-lactalbumin at 14,200 Daltons. These molecular weight markers tell little about the molecular weight of the antibiotic, except that it is quite small. Rather, the antibiotic was similar in molecular weight to bacitracin (Lane 4), which is a cyclic peptide antibiotic with

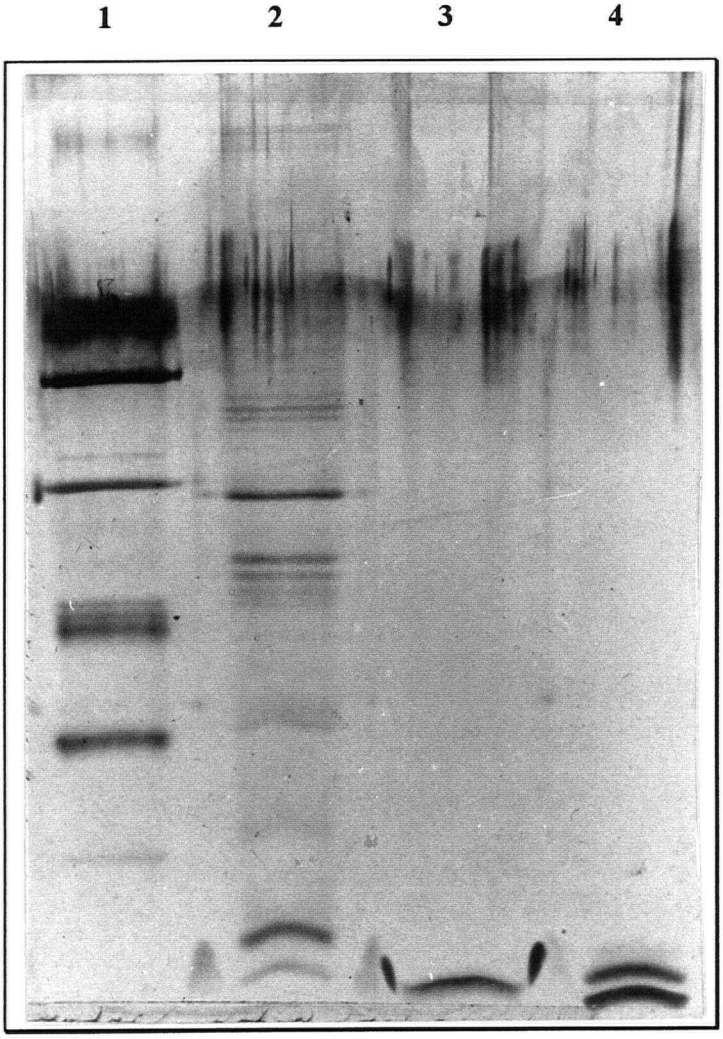


Figure 4.9: SDS PAGE of antibiotic. Lane 1 = MW standards (BSA - 66 kDa, Porcine Heart Fumarase - 48.5 kDa, Carbonic Anhydrase - 29 kDa, β-lactoglobulin - 18.4 kDa, α-lactalbumin - 14.2 kDa). Lane 2 = Culture supernatant. Lane 3 = Working form of antibiotic dissolved directly in loading buffer. Lane 4 = Bacitracin (1.5 kDa)

a molecular weight of 1,486 Daltons. The antibiotic in question was slightly larger than bacitracin which was assumed to be the lower of the two bands in lane four. Based on these findings the molecular weight of the antibiotic in question is approximately 1,500 Daltons.

The working form of the antibiotic (Lane 3) was free of any contaminating material as evidenced by the lack of any other bands in the lane. As a comparison, the culture supernatant (Lane 2) contained a lot of contaminating material. Evidently, all of this material was successfully removed by the purification protocol. These findings further confirm the purity of the working form of the antibiotic and are a qualitative tribute to the effectiveness of the purification protocol.

Figure 4.10 is the direct activity assay of a sample of culture supernatant that was run in an outside lane of the gel seen in Figure 4.9 and cut out prior to staining with silver. The section of gel was fixed, washed, and overlaid with soft agar seeded with *Agrobacterium vitis*. The plate was incubated overnight and the resulting zone of inhibition corresponded to the same position as the lone band in lane three (Figure 4.9). This suggests that the band in lane three (Figure 4.9) is in fact the antibiotic. Interestingly the antibiotic did not lose its activity when subjected to the reducing conditions of SDS PAGE.

Figure 4.11 reveals the results of a second gel. All lanes were run under the same conditions as the previous gel with the exception of lane 3. As a result lanes 1,2, and 4 are very similar in both Figures 4.9 and 4.11. The same sample was run in lane 3 in both gels (Figures 4.9 and 4.11) with one small exception. In Figure 4.9 the antibiotic was weighed

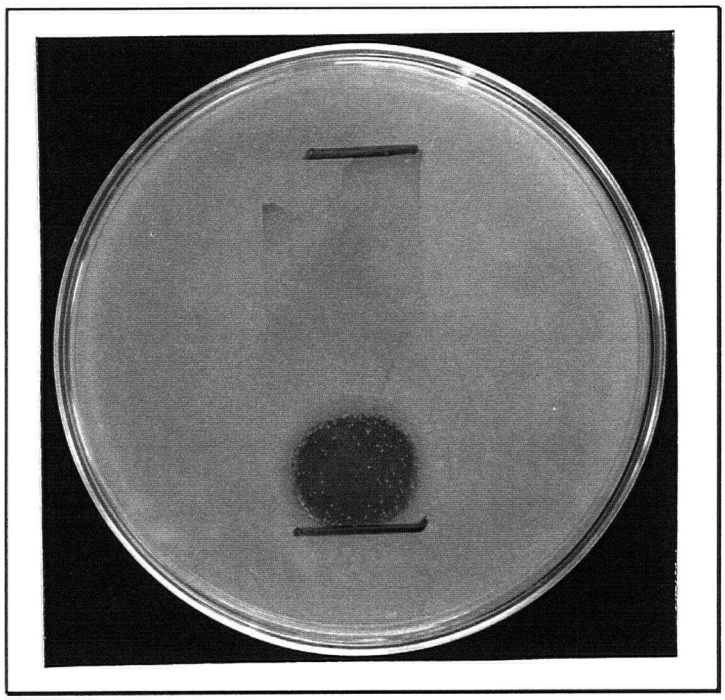


Figure 4.10: Direct activity assay of antibiotic on SDS polyacrylamide gel. Culture supernatant was run in an outside lane; the lane was cut out, fixed, washed, and overlaid with soft agar seeded with *Agrobacterium vitis.* The plate was incubated overnight.

and diluted directly in SDS PAGE loading buffer, while in Figure 4.11 the antibiotic was dissolved in distilled water prior to diluting in loading buffer. Both samples were loaded onto their respective gels at the same concentration and the same antibiotic preparation was used in each case. As a result of dissolving the antibiotic in water prior to diluting with loading buffer (Figure 4.11), a second band appeared. The second band corresponded with a molecular weight of approximately 20,000 Daltons and was significantly more concentrated than the smaller band which was quite faint and hard to see.

The apparent increase in the molecular weight is best explained by aggregation of the antibiotic. Since the sample is essentially pure, the antibiotic must have aggregated with itself perhaps to form micelles. In the first gel (Figure 4.9) the antibiotic was dissolved directly in the SDS containing loading buffer and the aggregates probably never had a chance to form. In the second gel (Figure 4.11), aggregates likely formed when the antibiotic was dissolved in distilled water. Once formed, the aggregates seemingly were not disrupted by diluting the antibiotic in the SDS containing loading buffer.

It is apparent from these results that the antibiotic formed stable aggregates when introduced into an aqueous environment in the absence of SDS. The formation of aggregates by antibiotics is a common occurrence, especially for peptides that demonstrate surface active properties. Examples include surfactin (Arima et al., 1968), arthrofactin (Morikawa et al., 1993), and biosurfactants produced by *Bacillus licheniformis* (Lin et al., 1994; Jenny et al., 1991). All of these compounds are lipopeptides, demonstrate surface active properties, and form high molecular weight aggregates. Mulligan and Gibbs (1990) took advantage of this last property in a one step method to purify and concentrate

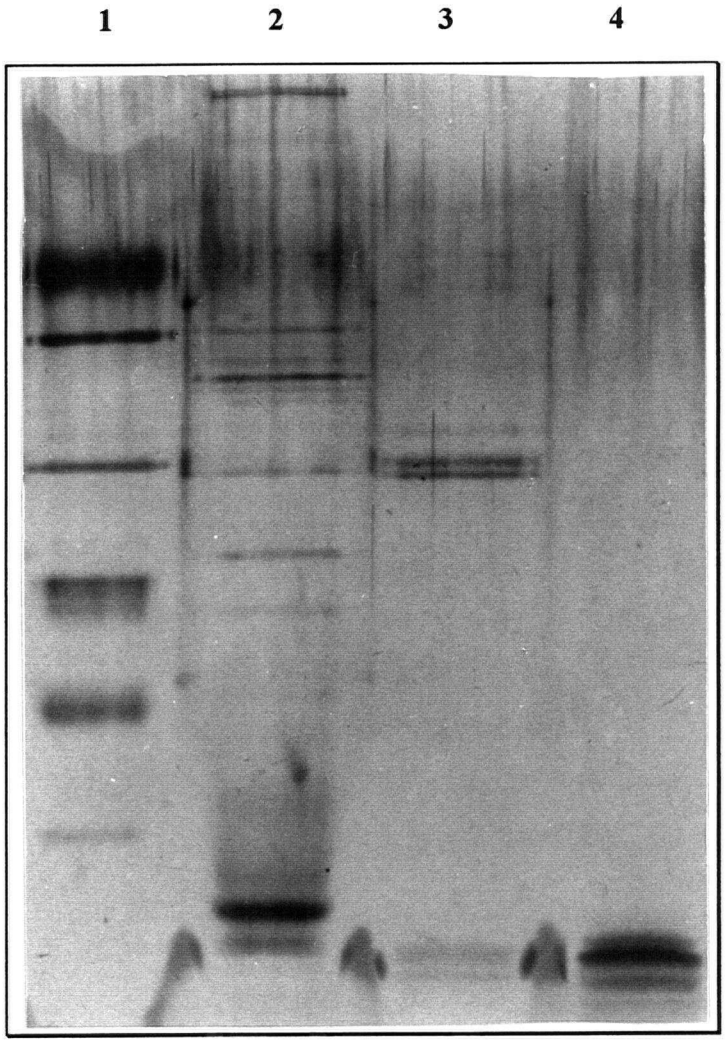


Figure 4.11: SDS PAGE of antibiotic. Lane 1 = MW standards (BSA - 66 kDa, Porcine Heart Fumarase - 48.5 kDa, Carbonic Anhydrase - 29 kDa, β- lactoglobulin - 18.4 kDa, α- lactalbumin -14.2 kDa). Lane 2 = Culture supernatant. Lane 3 = Working form of antibiotic dissolved in distilled water and then diluted in loading buffer. Lane 4 = Bacitracin (1.5 kDa).

surfactin from the culture supernatant by ultrafiltration. The ability of surfactant molecules to form micelles allows them to be retained by relatively high molecular weight cut-off membranes.

Surface active properties were demonstrated qualitatively for the antibiotic in this study (data not shown). When samples of the antibiotic (1 mg/ml) 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. This result is indicative of a notable decrease in the surface tension of the water containing the antibiotic. When distilled water containing none of the antibiotic was dropped onto the same surface, droplets formed and these could not be disrupted. The fact that the antibiotic forms aggregates and demonstrates surface active properties is further evidence that it belongs to the class of fatty acid containing peptolides often referred to as biosurfactants.

b. Gel filtration

Gel filtration of the antibiotic was accomplished using Biogel P-10 as the column matrix. Early attempts at gel filtration used a Sephadex matrix; however, the antibiotic demonstrated an affinity for the Sephadex (data not shown) and eluted beyond the column volume. Figure 4.12 presents a typical gel filtration chromatogram of the purified antibiotic on Biogel P-10.

Biogel P-10 effectively separates compounds between 1,500 and 20,000 Daltons. These limits are too broad to accurately determine the molecular weight of the antibiotic, since it is known that its molecular weight is approximately 1,500 Daltons. However, the matrix was not chosen as a means to obtain an accurate determination of molecular

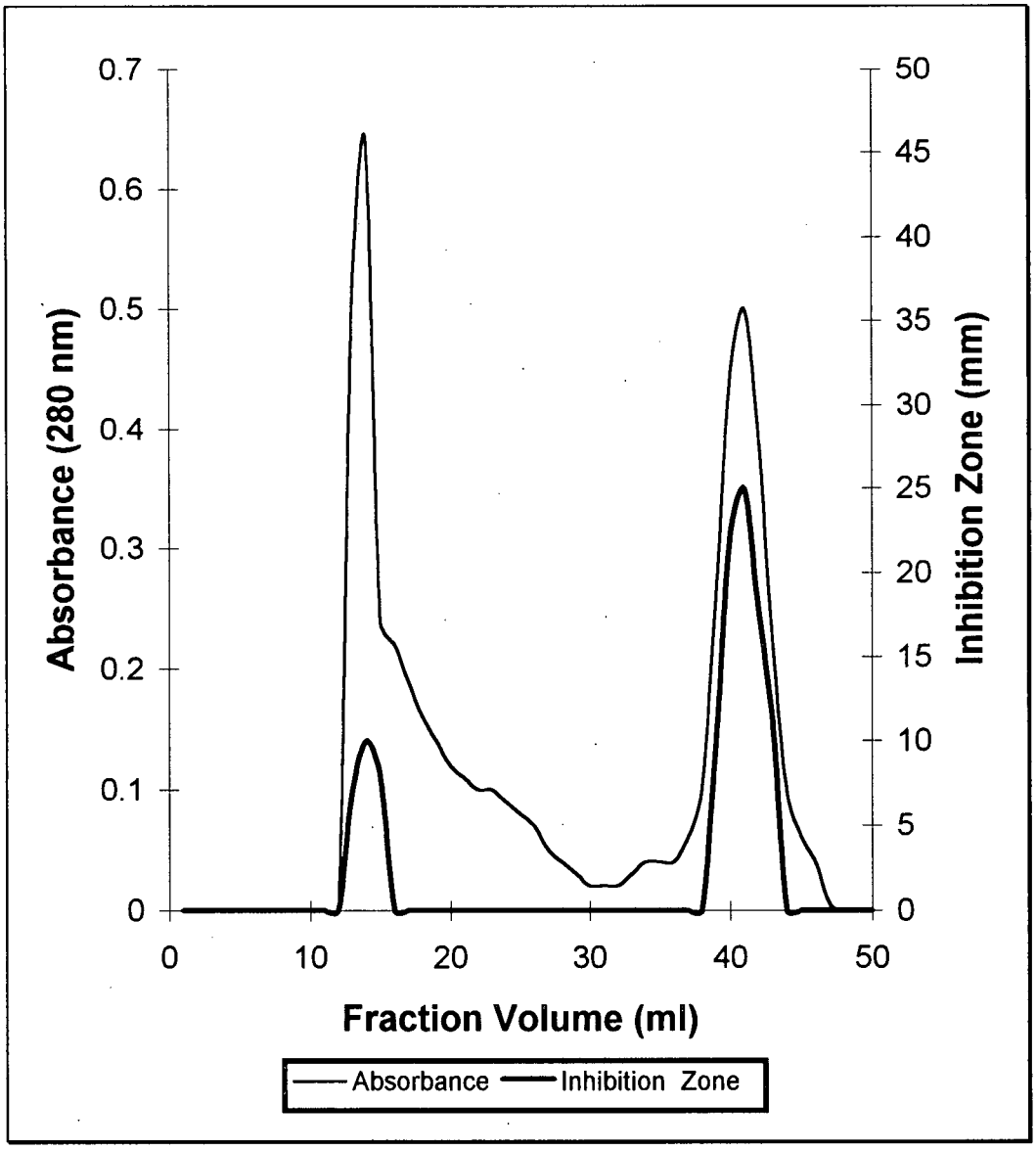


Figure 4.12: Elution pattern of antibiotic from a Biogel P-10 column equilibrated in 50 mM Tris buffer, pH 7.5. Void volume = 14 ml (Blue Dextran), column volume = 45 ml (copper sulfate). Molecular weight standards - streptomycin (1457 Da), and polymyxin B (1300 Da) eluted at 43 ml and 44 ml respectively.

weight, but rather to demonstrate that the antibiotic forms aggregates.

Figure 4.12 demonstrates that the antibiotic eluted from the column at two different volumes, indicating that it had two different molecular weights. Based on the elution volumes of the two standards (streptomycin and polymyxin), the low molecular weight form of the antibiotic (elution volume = 42 ml) appears to have a molecular weight of approximately 1,500 Daltons. See Figure 4.12 for elution volumes and molecular weight values for streptomycin and polymyxin. The antibiotic also eluted from the column at the void volume (14 ml), suggesting that it was also present as an aggregate with a molecular weight in excess of 20,000 Daltons. These results are in agreement with those from SDS PAGE (Figure 4.9 and 4.11), and they strengthen the hypothesis that the antibiotic forms aggregates in an aqueous environment.

E. Antimicrobial Characterization

1. Appearance of the Antibiotic in the Fermentation Broth

Figure 4.13 reveals the production of the antibiotic over the course of a typical fermentation. Cell growth was measured as optical density (600 nm) and activity of the cell free supernatant was measured as the zone of inhibition in the agar diffusion assay. It appears that the production of the antibiotic was associated with the logarithmic growth phase of the producing organism (*Bacillus subtilis*). The antibiotic first appeared in the culture supernatant at the beginning of log phase and its production ended near the beginning of stationary phase.

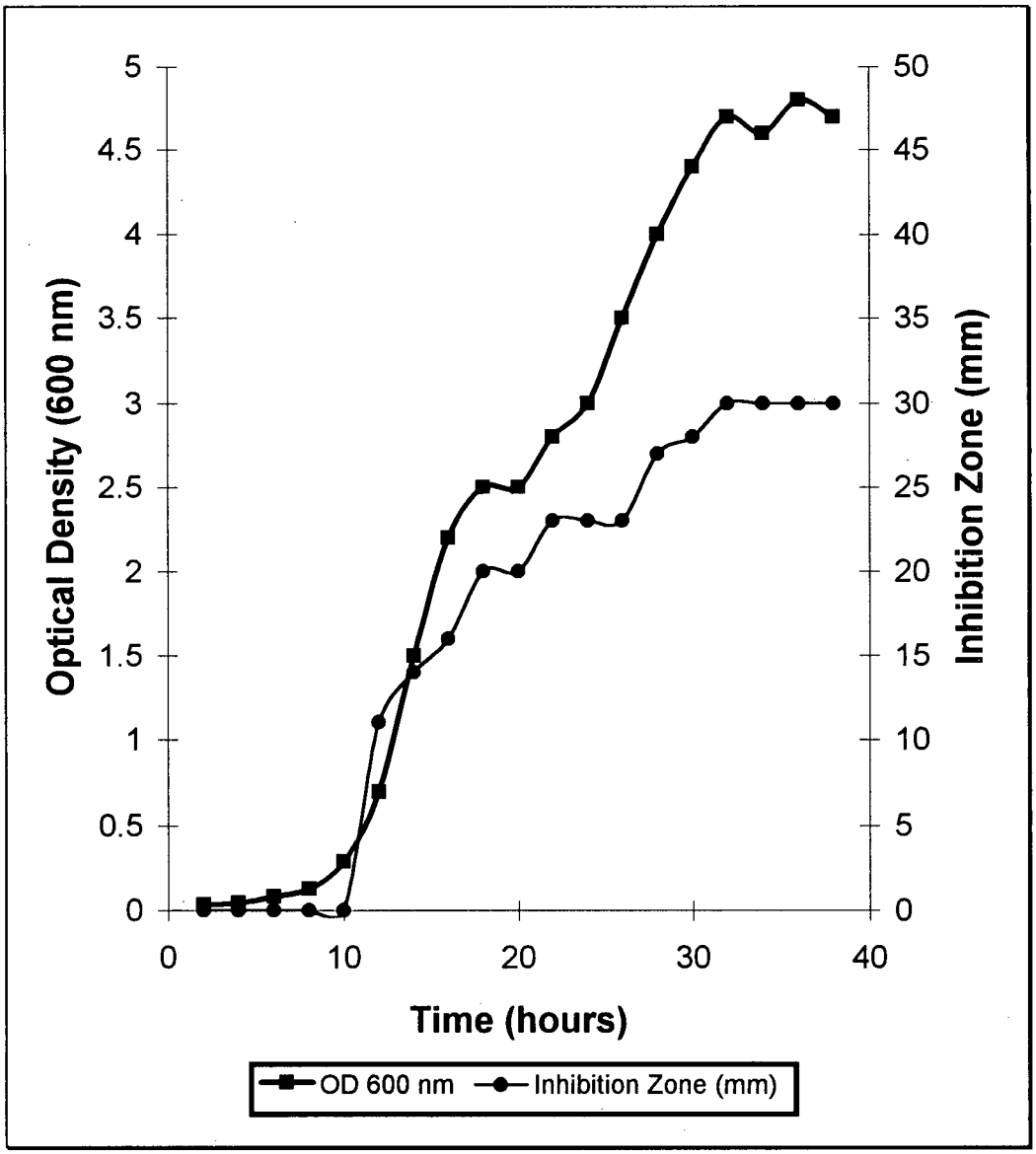


Figure 4.13: Appearance of the antibiotic in the culture broth over the course of a typical fermentation.

According to Katz and Demain (1977), the production of peptide antibiotics by *Bacillus* is usually initiated after the organism has passed the rapid growth phase. The results of this study are somewhat contradictory to the statement of Katz and Demain (1977), because the production of the antibiotic appears to coincide with active growth of the producing organism (*Bacillus subtilis*). Examples of other antibiotics produced during active growth of the producing organism include surfactin (Cooper et al., 1981), subtilysin (Bernheimer and Avigad, 1970), and the lipopeptide biosurfactant of *Bacillus licheniformis* JF-2 (Lin et al., 1994). Interestingly, each of these three compounds is a biosurfactant (demonstrates surface active properties), is a lipopeptide, and is produced by a species of *Bacillus*.

2. Activity spectrum

Twelve different organisms were assayed for their sensitivity to the antibiotic using the agar diffusion assay; the outcome is shown in Table 4.5. All of the Gram negative bacteria tested were sensitive to the antibiotic, albeit at varying degrees. The most sensitive Gram negative bacteria was *Agrobacterium 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 *Botrytis cinerea* seemed to be susceptible.

A typical picture of the agar diffusion assay for two of the organisms tested is shown in Figure 4.14. This picture clearly shows how sensitive *Agrobacterium vitis* was to the antibiotic. A large halo of growth inhibition surrounded the well in the agar plate. Interestingly, the same is not true for the fungi tested. *Botrytis cinerea* clearly was sensitive to the antibiotic; however, the effect was slightly different. *Botrytis cinerea* spores were able to germinate within the halo of growth inhibition, yet substantial mycelial development did not occur.

Table 4.5: Sensitivity of various organisms to the antibiotic.

Organism	Sensitivity
Escherichia coli (0157:H7)	+
Salmonella typhimurium	+
Erwinia amylovera	++
Pseudomonas corrugata	++
Pseudomonas fluorescens	+
Pseudomonas putida	+
Agrobacterium vitis (CG 1005)	+++
Staphylococcus aureus	-
Listeria monocytogenes	-
Bacillus cereus	-
Penicillium expansum	-
Botrytis cinerea	++

(-) = 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

3. Effect of the Antibiotic on the Growth of *Agrobacterium vitis*

Actively growing broth cultures of *Agrobacterium vitis* were treated with different concentrations of the antibiotic and assayed over the course of a few hours for either

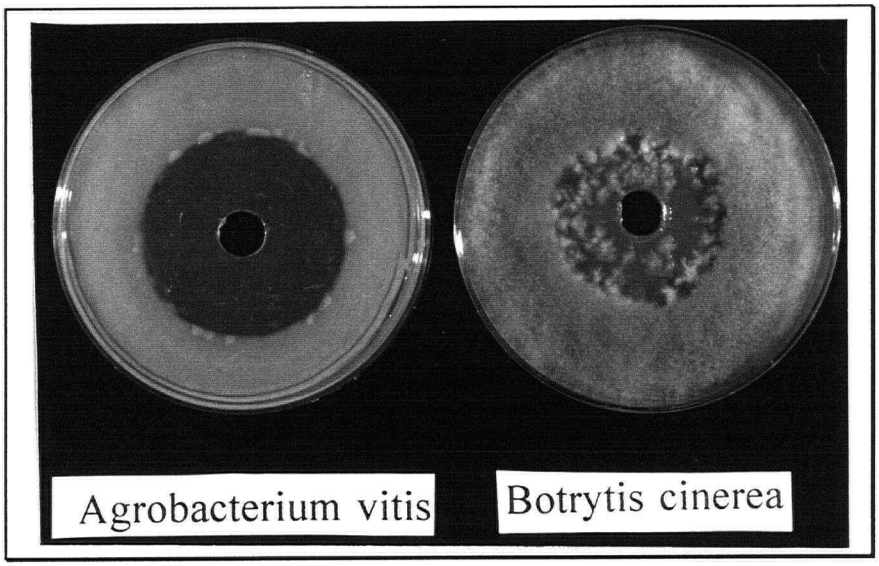


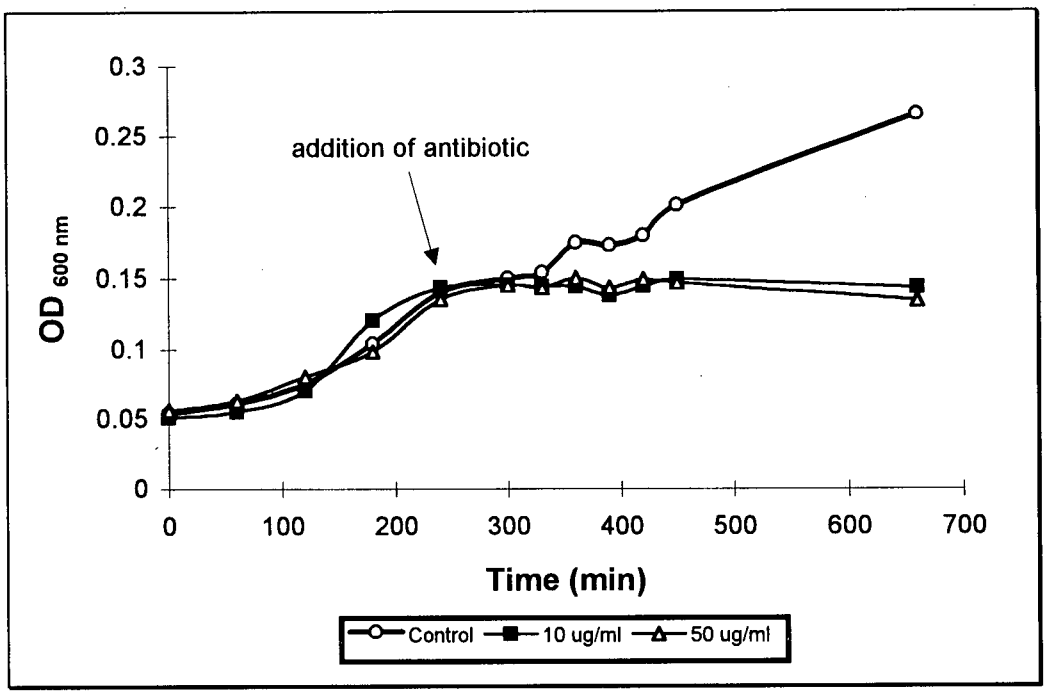
Figure 4.14: Agar diffusion assay. Demonstration of the sensitivity of *Agrobacterium vitis* and *Botrytis cinerea* to the antibiotic.

optical density (Figure 4.15 A) or for viable cells (Figure 4.15 B).

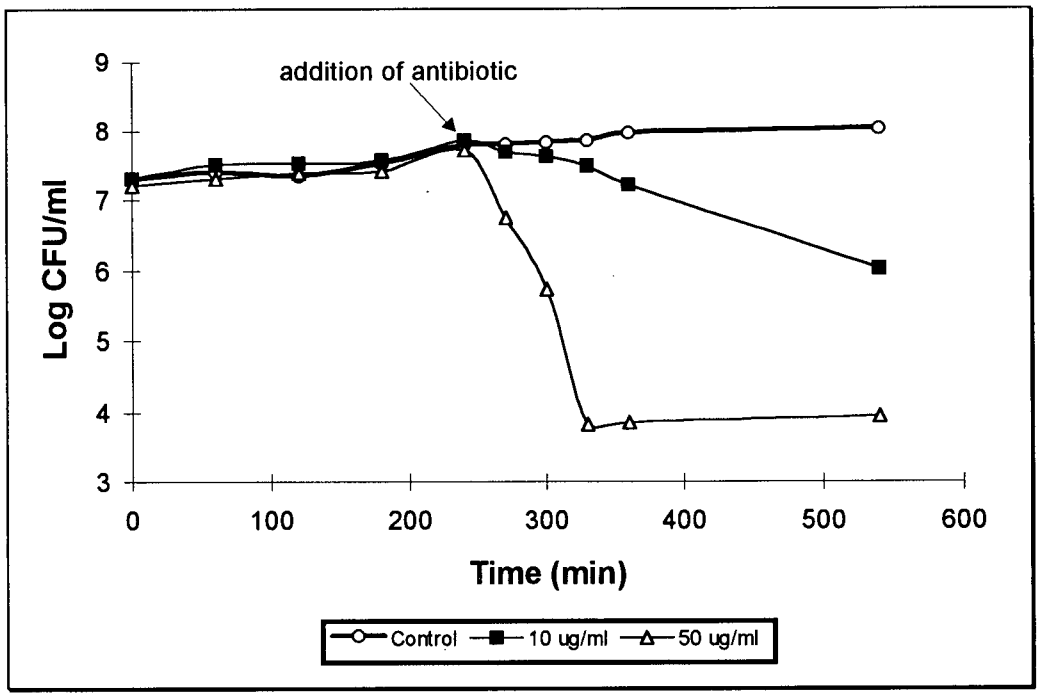
Figure 4.15 A shows the effect of the antibiotic on the integrity of the sensitive cells. The antibiotic stopped the growth of *Agrobacterium vitis* at each of the concentrations tested. Interestingly, no reduction in the optical density was observed, but rather the growth curves for the treated cells leveled off after treatment with the antibiotic. This suggests that the growth inhibition of *Agrobacterium vitis* occurred in the absence of subsequent cell lysis. According to Gonzalez et al. (1994) cell lysis is associated with a decrease in the optical density of the culture.

Figure 4.15 B demonstrates that the mode of action of the antibiotic is bactericidal, rather than bacteristatic. At both concentrations of the antibiotic tested, there was a notable decrease in the viable counts of the actively growing cultures after treatment. After 5 hours, the viable count of the cells treated at 10 ug/ml decreased approximately 100 fold compared to the control, whereas the viable counts of the cells treated at 50 ug/ml decreased in excess of 10,000 fold. If the effect of the antibiotic were bacteristatic rather than bactericidal, one would expect to see the viable counts level off rather than decrease as dramatically as they did.

Based on these results, it appears as though the antibiotic actively kills *Agrobacterium vitis* cells; however, the cell death does not appear to be associated with lysis. In other words, the antibiotic appears to have a bactericidal effect on actively growing cultures of *Agrobacterium vitis*; however, cell integrity does not appear to be destroyed.



A



B

Figure 4.15: Effect of different concentrations of antibiotic on a broth culture of *Agrobacterium vitis*. Effect on the optical density of the culture broth (A). Effect on the viable cell count (B).

V. Conclusions

Based on SDS PAGE and HPLC analysis of the purified antibiotic, the purification protocol appears to have been successful; however, at the expense of yield. The greatest loss in the recovery of the antibiotic occurred during ion exchange purification and is likely a result of a loss of activity, rather than a physical loss of the antibiotic. This apparent loss of activity could possibly be reduced/eliminated if the pH of the elution buffer were decreased and the NaCl gradient reduced, or if a pH gradient were used to elute the antibiotic from the column.

Amino acid analysis of the antimicrobial agent offers concrete evidence that the antibiotic is indeed a peptide. The antibiotic contains asparagine/aspartate, glutamine/ glutamate, serine, glycine, alanine, proline, valine, and leucine (2:3:1:1:1:1:1:4). The presence of an aromatic amino acid in the antibiotic was suggested by its ultraviolet absorbance; however this finding was not supported by the amino acid analysis.

Infrared spectroscopy of the antibiotic gives some interesting information about the antibiotic. The FTIR spectrum indicates the presence of a peptide, an acyl chain, and a lactone bond. The spectrum is nearly identical to that of several other lipopeptide antibiotics, which are often referred to as biosurfactants. More specifically these compounds are classified as fatty acid containing peptolides (lactone containing peptides). Further analysis (GC/MS) of the fatty acid portion of the antibiotic needs to be completed in order to confirm its presence and characterize its structure.

Molecular weight determinations (SDS PAGE and Gel filtration) suggest that the antibiotic forms aggregates in an aqueous environment. The antibiotic appears to have two

molecular weights; one corresponding to a monomer (approximately 1,500 Da), and one corresponding to the formation of an aggregate (approximately 20,000 Da). These observations offer further support to the idea that the antibiotic in question is best classified as a lipopeptide biosurfactant, all of which form aggregates and demonstrate surface active properties.

On the basis of empirical evidence, the antibiotic appears to demonstrate surface active properties. Surface active properties are associated with micelle formation and are closely related to the polar/hydrophobic characteristics of the antibiotic. The surface active properties of the antibiotic warrant further study, as there is increasing interest in biodegradable, surface active compounds, produced from renewable resources.

The antibiotic demonstrates some 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 *Agrobacterium vitis* cells suggest a bactericidal mode of action rather than bacteriostatic effect; however, cell death does not appear to be associated with cell lysis. It would be interesting to determine whether the antibiotic had similar effects on other sensitive Gram negative organisms.

Antimicrobial activity appears to be associated with active growth of the producing organism rather than with stationary phase. Antimicrobial activity of the antibiotic is stable over a fairly wide range of pH and temperature. The antibiotic activity begins to decrease at alkaline pH, but is stable at neutral and acidic pH. Complete loss of

activity is observed above pH 10. At an acidic pH (pH 3.0) the antibiotic begins to lose activity above 60°C, demonstrating a complete loss of activity at 100°C; however, at pH 7 the antibiotic remains stable up to 80°C and a complete loss of activity was not observed at 100°C.

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