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FURTHER PURIFICATION AND CHARACTERIZATION OF JENSENIIN P, A BACTERIOCIN PRODUCED BY PROPIONIBACTERIUM JENSENII B1264

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FURTHER PURIFICATION AND CHARACTERIZATION OF JENSENIIN P, A BACTERIOCIN PRODUCED BY *PROPIONIBACTERIUM JENSENII* B1264

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ABSTRACT

Jenseniin P, a bacteriocin discovered by Prince, can inhibit the growth of several strains of propionibacteria and lactobacillus(Prince 1993). Ratnam further purified jenseniin P by ammonia sulfate precipitation and anion exchange chromatography. In that study, partially purified jenseniin P was stable in various pH ranges, SDS concentrations, several solvents, and remained active after holding at 100 ° C for 60 minutes. The molecular weight was estimated to be between 6 and 9 kDa. In addition, jenseniin P was demonstrated to be inhibitory to *Propionibacterium acnes* using the spot-on-lawn method. Thus, Ratnam postulated its possible application in acne treatment(Ratnam 1997).

In this present work, jenseniin P was further purified using C18 resin silica column. Vivaspin and ammonia sulfate precipitation methods were also evaluated. This further purified jenseniin P was inactivated by trypsin, pronase, and protease K, which confirmed its proteinaceous nature. The molecular weight of jenseniin P was further narrowed down to be between 4.5 and 5kDa. Jenseniin P was characterized to be pH stable and heat stable using a microtiter plate method developed in this work, which was 8-fold more sensitive than the previous critical dilution method described by Ratnam. The mode of action of jenseniin P was determined to be bactericidal with a killing rate of 2.38 log h^{-1}.

We also detected a new agent produced by *Propionibacterium jensenii* B1264 that inhibited the growth of *Propionibacterium acnes* 6919 but not *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797, the indicator strain used in previous studies. This inhibitory agent was recovered from the 30% acetonitrile elution fraction of C18 resin silica column purification process, which was different from the previously reported jenseniin P, because the jenseniin P that inhibited the growth of *L. delbrueckii* subsp. *lactis* ATCC 4797 was mostly recovered from the 50% acetonitrile elution fraction. This *P. acnes* specific inhibitory agent is currently being investigated.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER ONE

LITERATURE REVIEW

1.1 Bacteriocin

1.1.1 General Characteristics of Bacteriocin

Antimicrobial peptides are produced by various kinds of organisms(Faye, Brede et al. 2002). Multiple antimicrobial peptides have been isolated from different organisms, such as mammals(Lehrer, Lichtenstein et al. 1993), amphibians(Zasloff 1987), fish(Cole, Weis et al. 1997), insects(Steiner, Hultmark et al. 1981), plants(Cammue, De Bolle et al. 1992), and different microorganisms(Jack, Tagg et al. 1995). In 1925, Gratia demonstrated that strain V of *Escherichia coli* can produce a dialyzable and heat-stable antimicrobial agent that inhibits the growth of *E. coli* ϕ, which was later named colicin V(Gratia 1925; Jack, Tagg et al. 1995). A whole series of colicins produced by *E. coli* and closely related members of the *Enterobacteriaceae* were discovered afterward. To date, most of the significant progress in bacteriocin research stemmed from study of the colicins, and this resulted in considerable in-depth knowledge of the genetic basis, domain structure, mode of formation, and inhibitory action of bacteriocins(Cotter, Hill et al. 2005).

By definition, bacteriocins are gene-encoded, ribosomally-synthesized proteins or peptides that usually display antimicrobial activity against species closely related

to the producer strains(Brede, Faye et al. 2004). Many bacteriocins produced by Gram-positive bacteria have been isolated and characterized(Faye, Brede et al. 2002), and most characterized bacteriocins thus far show a narrow spectrum of inhibition(Faye, Langsrud et al. 2000). However, there are exceptions. For example, some bacteriocins produced by lactic acid bacteria (LAB), such as nisin, inhibit not only closely related species but also food-borne pathogens such as *Listeria monocytogenes* and many other Gram-positive spoilage microorganisms.

1.1.2 Bacteriocins and Antibiotics

Even though both bacteriocin and antibiotics have antimicrobial activity and can be produced by bacteria, there are several differences between them. First, bacteriocins are synthesized via ribosome, while antibiotics are synthesized by enzymatic systems; Second, bacteriocins usually have a narrow spectrum of inhibition, while in general antibiotics have a much broader spectrum; Third, the gene that encodes the immunity protein of bacteriocin is always linked to the structure gene that encodes the bacteriocin, while the gene that determines antibiotic resistance is usually independent from the genes that encode the antibiotic synthesis apparatus(Dutton 2002). Antimicrobial peptides produced by bacteria are not necessarily bacteriocins. For example, the broad spectrum antimicrobial bacitracin is not a bacteriocin because its biosynthesis is carried out non-ribosomally by bacitracin synthetase ABC, a multi-enzyme complex(Froyshov 1977).

1.1.3 Classification of Bacteriocins Produced by LAB

The most thoroughly studied bacteriocins are bacteriocins produced by LAB because a wide variety of strains are routinely utilized as starter cultures in the manufacture of meat, dairy, and vegetable products(O'Sullivan, Ross et al. ; Nettles and Barefoot 1993). They cause rapid acidification of the raw material through the production of organic acids, mainly lactic acids. In addition, their production of ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes are of great value in food fermentation and safety.

It has been estimated that 3%-99% of bacteria and archaea can produce more than one bacteriocin; many of the reported bacteriocins are produced by LAB(Klaenhammer 1988). These bacteriocins are the most investigated and studied because they have immediate potential in food applications without being thoroughly purified. In 1993, based on common characteristics and structure, Klaenhammer proposed to classify all the discovered lactic acid bacteriocins into three main groups: Class I, II and III bacteriocins (Table 1) (Klaenhammer 1993; Nes, Diep et al. 1996)

Class I is composed of small, post-translationally modified peptides that are characterized by the presence of modified thioether amino acids and α-, β-unsaturated amino acids and are usually referred to as lantibiotics (Twomey, Ross et al. 2002). Class II is composed of a large group of heat-stable, unmodified peptide bacteriocins which can also be further subdivided to Class IIa, Class IIb and Class

IIc(Ennahar, Sonomoto et al. 1999). Class III bacteriocin consists of large, and heat-labile bacteriocins(Nes, Diep et al. 1996)

Table 1.1 Classification of LAB bacteriocin

Main category	Characteristics	Subcategory	Examples
Class I	Lantibiotics (containing lanthionine and β-lanthionine)	Type A (elongated molecules; molecular mass, <4 kDa)	Nisin A Nisin Z Subtilin Epidermin
		Type B (globular molecules; molecular mass, 1.8 to 2.1 kDa)	Mersacidin Actagardin Mutacin II
Class II	Nonmodified heat-stable bacteriocins containing peptides with molecular masses of <10 kDa	Subclass IIa (antilisterial pediocin-like bacteriocins)	
		Subclass IIb (two-peptide bacteriocins)	Plantaricin EF Plantaricin JK
		Subclass IIc (other peptide bacteriocins)	Lactococcin 972
Class III	Protein bacteriocins with molecular masses of >30 kDa		Helveticin J Millericin B

This table was reproduced with permission(Drider, Fimland et al. 2006), APPENDIX.

1.1.4 Applications of Bacteriocins

In recent years, there has been a significant focus on bacteriocins produced by Gram-positive bacteria. Many of these bacteriocins are food grade organisms that are already commonly used in food industry but now offer the further prospect of application to improve food preservation. Another contributing factor has been the burgeoning interest in possible applications of bacterial interference as a strategy for the prevention of certain infectious diseases(O'Sullivan, Ross et al.). In this present work, a bacteriocin produced by propionibacteria was studied. Examples of application of bacteriocins are described as follows:

First, bacteriocins can be used to enhance food quality. For example, a cheese made with a commercial starter culture that does not produce lacticin 3147 develops an undefined microflora while a cheese inoculated with the same commercial strain that produces lacticin 3147 and a resistant adjunct strain of lactobacillus, develops a single defined culture once the starter culture has died off, enabling the cheese manufacturer to control flora development (Fig 3a).

Second, bacteriocins may be produced directly in the food as a result of starter culture or co-culture activity[2]. Several studies have indeed indicated that LAB starter cultures or co-cultures are able to produce bacteriocins in food matrices, thus exhibiting inhibitory activity against sensitive food spoilage or pathogenic bacteria[2, 13]. A simple example of the usage of bacteriocins in food safety is the production of cottage cheese with a starter culture that produces a bacteriocin with activity against *Listeria monocytogenes* (Fig 3b).

Third, bacteriocins may be applied in veterinary medicine. For example, if a bacteriocin was incorporated into the teat seal and the teat was challenged with *Staphylococcus aureus*, the number of staphylococci recovered from 14 teats with bacteriocin is remarkably lower than those without bacteriocin (Fig 3c).

Fourth, the bacteriocin may be used to develop human medicine. A *Streptococcus mutans* strain that produces the lantibiotic mutacin, competitively excludes acidogenic *S. mutans*, thereby offering protection against tooth decay (Fig 3d).

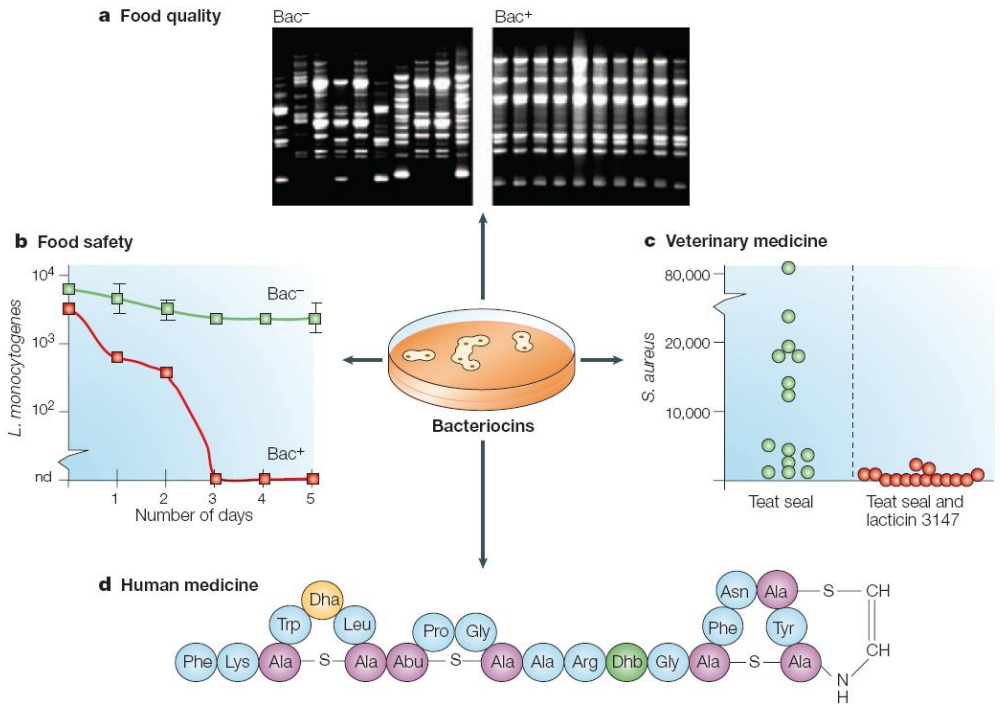

Figure 1.1 Selected applications of bacteriocins

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1.2 Propionibacterium

1.2.1 General Characteristics of Propionibacterium

According to Bergey's Manual of Determinative Bacteriology, 9th Edition, propionibacteria are non-filamentous pleomorphic rods, often clubshaped with one end rounded and other end tapered. Some of the cells might be bifid, coccoid or branched. Cells singly occur in short chains or pairs, in V or Y configurations or Chinese characters(Bergey and Holt 1994). They are nonmotile and nonsporing Gram-positive bacteria. They are facultative anaerobes, but most of them can grow somewhat in air, while better in anaerobic environment. They are found mainly in

cheese and dairy products fermentation producing large amounts of propionic and acetic acids and often small amount of gas. Based on their habitat, propionibacteria are divided into two groups, i.e., the classical propionibacteria and the cutaneous propionibacteria(Bergey and Holt 1994).

1.2.2 Classification of Propionibacterium

There are substantial differences between the two classes of propionibacteria. Classical propionibacteria were commonly found in dairy products, soil and olive fermentations. They can form eyes in Swiss chess because of CO_2 production and can produce vitamin B_{12} as well as propionic acid and bacteriocins(Vorobjeva 1999). The typical species are *P. freudenreichii, P. jensenii, P. thoenii, P. acidipropionici,* and *P. cyclohexanicum*(Vorobjeva 1999). The G+C content of classical propionibacteria is 65-67%. The second class is cutaneous propionibacteria, which typically includes *P. acnes, P. avidum, P. granulosum,* and *P. lymphophilum*. *P. acnes* is the major pathogen that causes inflammation in acne development(Yates 2005). The G+C content of cutaneous propionibacteria is 53-62%(Vorobjeva 1999).

1.2.3 Bacteriocins Produced by Propionibacteria

Many bacteriocins produced by propionibacteria have been described such as propionicin A produced by *P. freudenreichii*(Ramanathan, Wolynec et al. 1968), propionicin PLG-1 produced by *P. thoenii* P127(Lyon and Glatz 1991), but only four

are characterized on a molecular level. They are propionicin SM1 produced by *P. jensenii* DF1(Miescher, Stierli et al. 2000), propionicin T1 produced by *P. thoenii*(Faye, Langsrud et al. 2000), a precursor protein of an antimicrobial peptide (PAMP) produced by *P. jensenii* LMG3032(Faye, Brede et al. 2002), and propionicin F produced by *P. freudenreichii*(Brede, Faye et al. 2004). All of these four bacteriocins bacteriocins are initially synthesized as prebacteriocin first and then modified by different enzymes to form mature bacteriocins. These two characteristics are similar to those of the Class IIa bacteriocins produced by LAB(Drider, Fimland et al. 2006). The most recent characterized one was propionicin F. There are ten open reading frames (ORF) in the gene cluster, where prebacteriocin, protease, transposase and ABC transporter proteins are synthesized(Brede, Faye et al. 2004).

1.3 Acne

Common acne or acne vulgaris is a chronic inflammation of the sebaceous follicles that affects 85-100% of those aged 12-24(Burton, Cunliffe et al. 1971; Bershad 2001). The comedo, a plug of sebum and keratin lodged in the follicular duct, is its primary lesion. Comedones appear clinically as blackheads and whiteheads. It may persist in some individuals to later years. Although symptoms resolve in most individuals by the time they are 23 to 25 years of age, 1% of men and 5% of women still are affected at age 40(Cunliffe and Gould 1979). Although

not life-threatening, acne causes psychological and social distress and can cause profound scarring and disfiguring with adverse long term social consequences. Concern about acne is the most frequent reason that teenagers consult a physician(Goodman 1999).

1.3.1 Acne Pathogenesis

Three underlying factors are implicated in human acne. They include (1) increased secretion of sebum, an event controlled by genetic factors and stimulated by androgenic hormones, (2) excess formation of keratin resulting in plugging of the sebaceous duct, through which the sebaceous gland empties sebum into the sebaceous follicle and (3) growth of *P. acnes*. *P. acnes* is believed to play a pivotal role in converting mild acne to inflammatory acne (Table 1.2)(Bershad 2001; Dessinioti and Katsambas 2010).

Table 1.2 Data on the mechanism of action of *P. acnes* in acne pathogenesis

- *P acnes* produces lipases, proteases, hyaluronidases, and neutrophil chemotactic factors
- *P acnes* induces the production of TNF-α, IL-1α, and IL-8
- *P acnes* induces the expression of the proinflammatory cytokines IL-8, IL-1β, and TNF-α by human monocytes in acne patients and in controls
- Inflammation triggered through TLR2 is important in the pathogenesis of acne, and *P acnes* was shown to induce monocyte cytokine production (IL-12, IL-8) through a TLR2–dependent pathway
- An increase in TLR2, TLR4, and MMP-9 expression by human keratinocytes occurred with incubation with *P acnes* fractions
- *P acnes* extracts are directly able to modulate the differentiation of keratinocytes by inducing β1, α3, α6s, and αVβ6 integrin expression, and filaggrin expression on keratinocytes
- *P acnes* induces IL-8, and β-defensin-2 expression in keratinocytes via TLR2 and TLR4
- *P acnes* induces keratinocyte growth *in vitro*
- *P acnes* GroEL (a heat-shock protein) is able to upregulate the proinflammatory cytokine production of keratinocytes
- *P acnes* may be involved in the formation of the microcomedones
- *P acnes* biofilm may lead to the increased cohesiveness of corneocytes seen in acne

IL, interleukin; *MMP*, matrix metalloproteinase; *TLR*, toll-like receptor; *TNF-α*, tumor necrosis factor-α.

This table was reproduced with permission (Dessinioti and Katsambas 2010), APPENDIX.

1.3.2 Current Treatments of Acne

Acne is a chronic disorder and its response to treatment is relatively slow. Logical approaches for treatment of acne require targeting the above aetiological

factors are listed as following(Yates 2005).

Table 1.3 Current treatments of acne(Dessinioti and Katsambas 2010)

Aetiological factors	**Treatment**
sebaceous gland blockage	retinoid and benzoyl peroxide
bacterial colonization	antibiotics, benzoyl peroxide, azelaic acid
seborrhoea	isotretinoin, retinoid, antibiotics

1.3.3 Bacteriocin Inhibition of *P. acnes*

P. acnes have been routinely treated by antibiotics, but antibiotic resistance is an emerging problem in recent years(Yates 2005). Treating acnes with bacteriocins might be an alternative choice for avoiding antibiotic resistance. One bacteriocin, ESL5, produced in the supernatant of *Enterococcus faecalis* SL-5, showed antimicrobial activity against numbers of Gram-positive pathogens, especially *P. acnes*. In addition, cell-free culture supernatant of *E. faecalis* SL-5 was incorporated into a lotion for application on the patients in one clinic trial. The lotion with this bacteriocin significantly reduced the inflammatory lesions in mild to moderate acne because of its inhibition against *P. acnes*(Kang, Seo et al. 2009).

1.4 Previous Studies of Jenseniin P

In 1993, Prince obtained eight dairy cultures from The All Union Collection of

Microorganisms in Moscow and examined them for acid-unrelated inhibition of 62 Gram-positive and Gram-negative bacterial strains by the deferred agar spot-on-lawn detection methods(Prince 1993). The results showed that *P. jensenii* P126, inhibited itself, *P. acidipropionici* P5, *P. jensenii* P126, *P. thoenii* P127, *P. species* P1262 and P1263, *L. delbrueckii* ssp. *lactis* ATCC 4797, *L. delbrueckii* ssp. *lactis* ATCC 9649, *L. bulgaricus* NC1489 and *L. acidophilus* ATCC 4357. Among all the tested strains, *L. delbrueckii* ssp. *lactis* ATCC 4797, from here on referred to as *L. delbrueckii*, was the most sensitive strain. The inhibitory agent(s) was concluded by Prince to be a bacteriocin and was named jenseniin P(Prince 1993).

In 1997, Ratnam partially purified the jenseniin P by ammonium sulfate precipitation at 70% saturation and by batch anion exchange chromatography. Jenseniin P was characterized to be heat-stable, pH-stable, and bactericidal in her study. In that purification and characterization, *L. delbrueckii* was used as the indicator strain. In addition, Ratnam reported for the first time that jenseniin P, a third bacteriocin produced by dairy propionibacteria, was also an anti-acne bacteriocin that could inhibit the growth of *P. acnes*(Ratnam 1997). In her study, Ratnam presumed that the bacteriocin that inhibited the growth of *P. acne* to be the same one that inhibited the growth of *L. delbrueckii*.

1.5 Objectives

The primary objective of this research is to further purify jensenii P. The second

objective is to confirm the proteinaceous nature of jenseniin P, determine its molecular weight, mode of action and test its pH and heat stability. The third objective is to determine whether the bacteriocin that inhibits the growth of *L. delbrueckii* is the same agent that inhibits the growth of *P. acnes* 6919.

CHAPTER TWO

FURTHER PURIFICATION OF JENSENIIN P

2.1 Introduction

Bacteriocins are antimicrobial proteins or peptides. Hence, most bacteriocins have been purified using various combined protein purification methods. The four genetically characterized bacteriocins produced by propionibacteria were purified in this manner with variations, depending on the characteristics of the bacteriocins.

Propionicin SM1, a bacteriocin produced by *P. jensenii* DF1, was purified first by cation-exchange column and second by gel filtration column. After several rounds of purification using these two methods, the sample was subjected to SDS-PAGE electrophoresis. The separated bands were transferred to a polyvinylidene difluoride Immobilon™ membrane. After electroblotting, the transferred propionicin was detected by protein staining. The protein was sequenced with gas-phase sequencing(Miescher, Stierli et al. 2000).

PAMP, produced by *P. jensenii* LMG3032, was precipitated by 40% ammonium sulfate (wt/vol) followed by a 3 ml cation-exchange column and by gel filtration column(Faye, Brede et al. 2002).

Propionicin T1, a bacteriocin from *P. thoenii* was first precipitated from the supernatant by the addition of 40% ammonium sulfate and then purified by a cation

exchange column followed by a reversed-phase chromatography(Faye, Langsrud et al. 2000).

Propionicin F, a bacteriocin produced by *P. freudenreichii* LMGT2946 was precipitated from the supernatant with 40% ammonium sulfate and then purified by an anion-exchange column followed by a reverse phase chromatography(Brede, Faye et al. 2004). In further details, the sample was applied onto the RESOURCE RPC 1 ml column first, followed by application onto a Source 5RPC ST 4.6/415 column and then a Sephasil peptide C8 5 µl ST 4.6/250 column. In each round, the peptide was eluted from the column in a water-2-propanol gradient containing 0.1% trifluoroacetic acid. The fractions showing the highest specific bacteriocin activity were pooled and used in subsequent purification steps(Brede, Faye et al. 2004).

In this present work, after removing *P. jensenii* cells by centrifugation and membrane filtration (pore size 0.45µm), the bacteriocin jenseniin P were concentrated by lyophilization. After being resuspended in sodium phosphate buffer, the sample was dialyzed with a membrane (3.5kDa molecular weight cutoff membrane) to remove small molecules. An important purification step was the C18 resin silica column, a reverse phase chromatography. Purification steps using Vivaspin and ammonia sulfate precipitation were evaluated, in which 100kDa and 3kDa molecular weight cutoff membranes and various concentration of ammonia sulfate were utilized to concentrate and purify jenseniin P.

2.2 Materials and Methods

2.2.1 Cultures and Media

P. jensenii B1264 and *L. delbrueckii* subsp. *lactis* ATCC 4797 were obtained from Dr. Susan Barefoot, Clemson University, South Carolina. *P. acnes* 6919 was purchased from American Type Culture Collection (ATCC, Manassas, VA).

P. jensenii B1264 was cultivated (1% inoculum) in sodium lactate broth (NLB), consisting of 1% trypticase soy broth without dextrose, 1% yeast extract, and 1% sodium lactate syrup. Sodium lactate agar (NLA) and soft NLA were prepared by adding 1.5% or 0.75% agar to NLB, respectively.

L. delbrueckii subsp. *lactis* ATCC 4797 was cultivated in lactobacilli MRS broth for 12 hours at 37℃. MRS agar and soft MRS agar were prepared by adding 1.5% or 0.75% agar to MRS broth, respectively.

Frozen stock cultures were maintained at -80℃ in the appropriate medium containing 15% glycerol. Working cultures were prepared from stock cultures by sub- culturing three times in the appropriate medium.

2.2.2 Bacteriocin Microtiter Plate Assay

Activity of bacteriocin in liquid media was measured in a microtiter plate assay(Faye, Brede et al. 2002). In brief, each well of the microtiter plate was filled with 50 µl of a two-fold serial dilution of the antimicrobial sample in NLB or

sodium phosphate buffer and 150 µl of overnight *L. delbrueckii* subsp. *lactis* ATCC 4797 in MRS broth (OD_{600} = 0.02). The plates were incubated at 37℃ for 12 h; growth inhibition was measured spectrophotometrically at 620 nm with a 96 well microtiter plate reader. By definition, 1 unit of antimicrobial activity (AU) causes 50% growth inhibition (50% of the turbidity of a control culture without the peptide)(Faye, Brede et al. 2002). The total antimicrobial unit was defined as the reciprocal of the highest dilution showing 50% inhibition of the indicator strain and was expressed in activity unit (AU)/ml.

2.2.3 Bacteriocin Critical Dilution Assay

Bacteriocin activity on plate was measured by the critical dilution method(Barefoot and Klaenhammer 1984). An MRS agar plate was overlaid with 5 ml soft MRS agar inoculated with 10 µl of overnight MRS broth culture, *L. delbrueckii* subsp. ATCC 4797. Serial twofold dilutions of antimicrobial samples were spotted onto the plate. 1 unit of jenseniin P was defined as the reciprocal of highest dilution showing clear zones of inhibition and total antimicrobial activity was expressed in activity units per milliliter (AU/ml).

2.2.4 Total Protein BCA Assay

The Thermo Scientific Micro BCA™ Protein Assay Kit (Mfr. No. 23252) was used to measure total protein. In brief, bovine serum albumin (BSA) was used to

prepare protein standards. 150 µl of standard or unknown sample was loaded into microplate wells and 150 µl of the Micro BCA working reagent was added to the wells and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37 ℃ for 2 hours. When the plate cooled down to room temperature, the absorbance at 570nm was measured on a plate reader. The average 570 nm absorbance reading of the blank standard was subtracted from all of the other individual standards and unknown samples. A standard curve was prepared by plotting the average blank corrected 570nm reading for each BSA standard vs. its concentration in µg/ml. The standard curve was used to determine the protein concentration of each unknown sample.

2.2.5 Cultivation of *P. jensenii* B1264

P. jensenii B1264 was refreshed from frozen culture in -80 ° C and subcultured two generations on NLA plate and NLB media. After checking purity with gram stain, the *P. jensenii* B1264 was inoculated to sodium lactate broth in a 2L bioreactor anaerobically at 30 ℃ for 18 days. The culture was fed sodium lactate syrup (60%) twice daily to provide a 0.3% concentration of sodium lactate by volume. The culture was monitored daily for optical density (600nm), CFU count, pH, and antimicrobial activity of the supernatant against *L. delbrueckii* subsp. *lactis* ATCC 4797. The antimicrobial activity was measured using the microtiter plate method. The supernatant was harvested at day 18 for further purification.

2.2.6 Concentration of Jenseniin P

The culture was centrifuged at 10,000 x g for 30 min at 4℃. The supernatant was filtered by membrane (pore size 0.45μm). The supernatant was then concentrated by lyophilization (-80℃, overnight) and was resuspended in 0.05M sodium phosphate buffer. 1 ml of the sample was dialyzed (3500MWCO membrane) two times against 12 L of 0.05M sodium phosphate buffer overnight, and the buffer was changed twice.

2.2.7 C18 Resin Silica Column Purification of Jenseniin P

0.5g C-18 resin silica gel (360130500 Acros Organics) was used to bind protein from the sample. The protein was eluted from the resin with increasing concentrations of acetonitrile (015-4 4L Burdick & Jackson) plus trifluoroacetic acid (TFA) (Sigma T6508 10 Amps). Acetonitrile dilutions were prepared and the sample volume was increased by dilution with ultrapure distilled H_2O (Invitrogen 10977-015). In brief, 0.5g C-18 resin was loaded into a plastic disposable column (Pierce cat# 29920) and fixed to allow protein binding. First, the column was washed by adding 5 ml of 5% acetonitrile solution (5% acetonitrile, 94.9% ultrapure DI H_2O, 0.1% TFA), then by 100% acetonitrile solution (99.9% acetonitrile, 0.1% TFA), and again by 5% acetonitrile solution. Second, a 1 ml sample was loaded onto the resin and incubated by shaking on an ice bed for 1 hour. Unbound protein from the column material was eluted by 5 ml of sodium phosphate buffer (0.05M, pH 6.4),

and elution was collected. Five ml of 10% acetonitrile was used to wash the resin and the elution was saved in a 15 ml conical screw cap tube. The resin was washed with 20%, 30%, 40%, 50%, and 100% acetonitrile in the same method and the samples were collected separately in the appropriately labeled tubes. All samples, including an unbound sample, 10%, 20%, 30%, 40%, 50%, and 100% acetonitrile elution samples were frozen to -80 ℃ then freeze-dried overnight. Freeze-dried samples were then resuspended in the original volume of sample.

2.2.8 SDS-PAGE Analysis

Three identical 25 µl of partially purified jenseniin P were loaded onto an 18% SDS-PAGE gel and 10 µl of low-range rainbow molecular weight marker (Amersham Biosciences, RPN 755) were loaded into a separate lane. The gel was run at a constant voltage of 100V for 45 min. The gel was then washed in 200 ml of 2.5% Trion X-100 for one hour and then in 200 ml of sterile distilled water for 4 h with gentle shaking(Miescher, Stierli et al. 2000). The gel was overlaid with 0.75% soft MRS agar inoculated by *L. delbrueckii* subsp. *lactis* ATCC 4797. The gel was incubated at 37℃ overnight. The position of the clear zone of inhibition on the indicator overlay was compared to that of the marker to determine the molecular weight of the jenseniin P in the sample.

2.3 Results and Discussion

2.3.1 Comparison of Two Bacteriocin Bioassay Methods

Jenseniin P was produced in a 2L bioreactor. Five daily samples of supernatant (day 9, 10, 11, 12, 14) from the second batch of bioreactor samples were assayed using both the microtiter plate method and the critical dilution method. The results showed that the sensitivity of the microtiter plate method was 8-fold more sensitive than the critical dilution method (Fig 2.1).

Both of the method depended on 2-fold dilution of samples. But the microtiter plate method is more accurate and sensitive because the result was quantified by a spectrophotometer, while the critical dilution method depended on the evaluation by naked eyes. One 96-well plate can hold 8 samples while one petri dish can only be used for one sample. The disadvantage of the microtiter plate method was that at least 100 µl of sample was required in one single set of assay which was more than that of the critical dilution method. However, due to the higher sensitivity of the microtiter plate method, a more diluted sample could be utilized.

In the purification of jenseniin P, the activity of samples after each purification step was quantified by the microtiter plate method. Because it is convenient, accurate, and sensitive, meaningful statistical analysis can be carried out.

2.3.2 Production of Jenseniin P

Four batches of *P. jensenii* were produced in the bioreator. In the first batch, OD_{600}, bioactivity against *L. delbrueckii*, and pH were monitored daily from day 0 to day 33. The purpose was to determine that when the activity of jenseniin P reached the maximum. The result showed that the bioactivity became detectable from day 7 in the middle exponential phase and remained stable from day 12 to day 17 in the early stationary phase and decreased between day 17 and 33, which was from the middle to late stationary phases (Fig 2.2). In order to harvest active jenseniin P, the supernatant should be collected near day 17. This result is consistent with bacteriocin production by other propionibacteria: propionicin T1(Faye, Langsrud et al. 2000), propionicin SM1(Miescher, Stierli et al. 2000), a precursor protein of an antimicrobial peptide (PAMP)(Faye, Brede et al. 2002), and propionicin F(Brede, Faye et al. 2004) are all collected in the late exponential phase or early stationary phase for further purification. The reason for this result may be that when the nutrients were limited in the late stationary phase, the bacteriocin was used to inhibit growth of other competiters, thus the amount of bacteriocin in the supernatant started to decrease.

In the second batch, the OD_{600}, CFU, bioactivity, and pH were monitored daily. Since the activity of supernatant reached its maximum level between day 14 and day 17, the supernatant was harvested on day 18 in the second batch(Fig 2.3).

Since the CFU reached its maximum on day 3 and maintained at the same level

while the OD continued to increase till day 14, it indicated that jenseniin P is probably produced in middle stationary phase.

In the third batch, on day 5, the bacth culture was found to be contaminated accornding to result of gram stain (data not shown), because some bacteria did not show the typical cell morphology of *P. jensenii*. The culture could be easily contaminated since samples were collected daily.

In the fourth batch, the OD, CFU, bioactivity, and pH were monitored daily. The OD was lower than previous batches and there was no detectable bioactivity (Fig 2.4). The possible reason was that when single colonies were picked out for subculturing, the jenseniin P defective cells were picked out and inoculated to the 2L fermenter. This strain was preserved to be further characterized.

In the cultivation of *P. jensenii*, sodium lactate syrup was fed into the media daily instead of one time at the beginning, because the initial high concentration of sodium lactate has been shown to be toxic to *P. jensenii*(Wallin 2000). Continuous culture method was not chosen due to the fact it might keep the bacteria in exponential phase rather than the stationary phase when the bacteriocin is being produced.

2.3.3 Purification Result of Jenseniin P

The *P. jensenii* cells were removed by centrifugation, because activity was detected in the supernatant. The remaining cells in the supernatant were filtered

using membrane filtration (pore 0.45 um). The supernatant was then concentrated by lyophilization and was resuspended in sodium phosphate buffer. The final volume was adjusted to 1/20 of its original volume. The advantage of lyophilization was that the temperature was low and it would not denature the protein. Another advantage was that the sample could be resuspended in any volume with no lost of solute. The sample was dialyzed to remove soluble constituents and substances with a molecular weight less than 3.5kDa in the sample.

After dialysis, the sample was loaded to C18 resin silica column for reverse phase chromatography. During the C18 purification process, each acetonitrile elution fraction was collected separately. The 50% acetonitrile elution fraction had the highest activity against *L. delbrueckii*, while other fractions showed significantly lower activity (Fig 2.5).

The bioactivity after each purification step was shown (Table 2.1). Compared with the supernatant of day 18, the specific activity increased 7.81 fold after lyophilization, and the specific activity increased 19.04 fold in the 50% acetonitrile elution fraction after C18 resin silica column. The result indicated that the purification process was effective. .

After C18 purification, each individual fraction was loaded onto an SDS-PAGE gel and stained with silver stain. Two prominent bands with molecular weight ranging from 3.5 to 6.5kDa markers were observed in the 50% acetonitrile elution fraction (Fig 2.6).

The principle of C18 reverse phase chromatography purification is based on the polarity of proteins. When proteins bind to the non-polar C18 matrix, polar proteins bind loosely while non-polar proteins have high affinity to C18. Since acetonitrile is a non-polar mobile phase, polar proteins were eluted by lower percentage of acetonitrile solution, while non-polar proteins were eluted by higher percentage of acetonitrile. Jenseniin P was mainly eluted in the 50% acetonitrile elution fraction. In comparison to propionicin T1 and propionicin F, they were eluted using a continuous mobile gradient(Faye, Langsrud et al. 2000; Brede, Faye et al. 2004). In order to further purify jenseniin P, subsequent purification process should utilize an acetonitrile gradient.

In preliminary studies, a Vivaspin method was used in an attempt to concentrate proteins with a molecular weight between 3 and 100kDa. The problem with Vivaspin was that the pores in the membrane were easily clogged by large molecules. Ammonia sulfate solution was also attempted to precipitate jenseniin P. The activity was detected in 60%, 70% and 80% ammonia sulfate solution, but none of the fractions showed significant high activity. In comparison, propionicin T1 and propionicin F had been precipitated by 40% ammonia sulfate solution(Faye, Langsrud et al. 2000; Brede, Faye et al. 2004). Since the higher the ammonia sulfate concentration, the stronger the ionic strength, this suggested that jenseniin P was less polar than propionicin T1 and propionicin F.

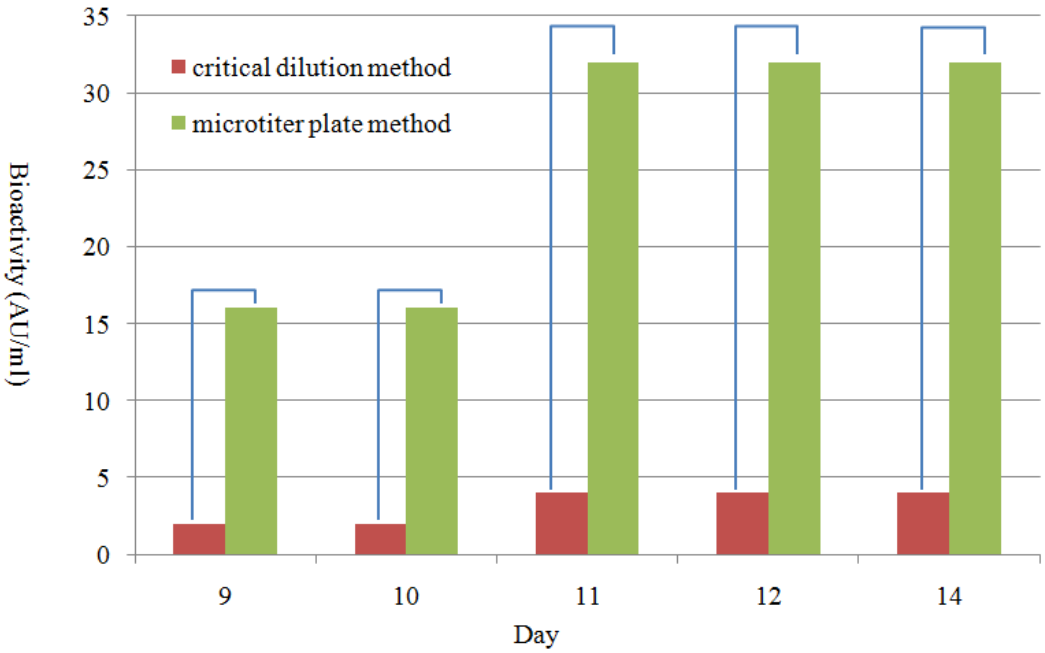

Figure 2.1 Comparison of two bacteriocin bioassays on 5 daily samples, n = 3. Standard deviations in each daily samples are all zero; the microtiter plate method is 8-fold more sensitive than the critical dilution method. Bracket indicates significant difference.

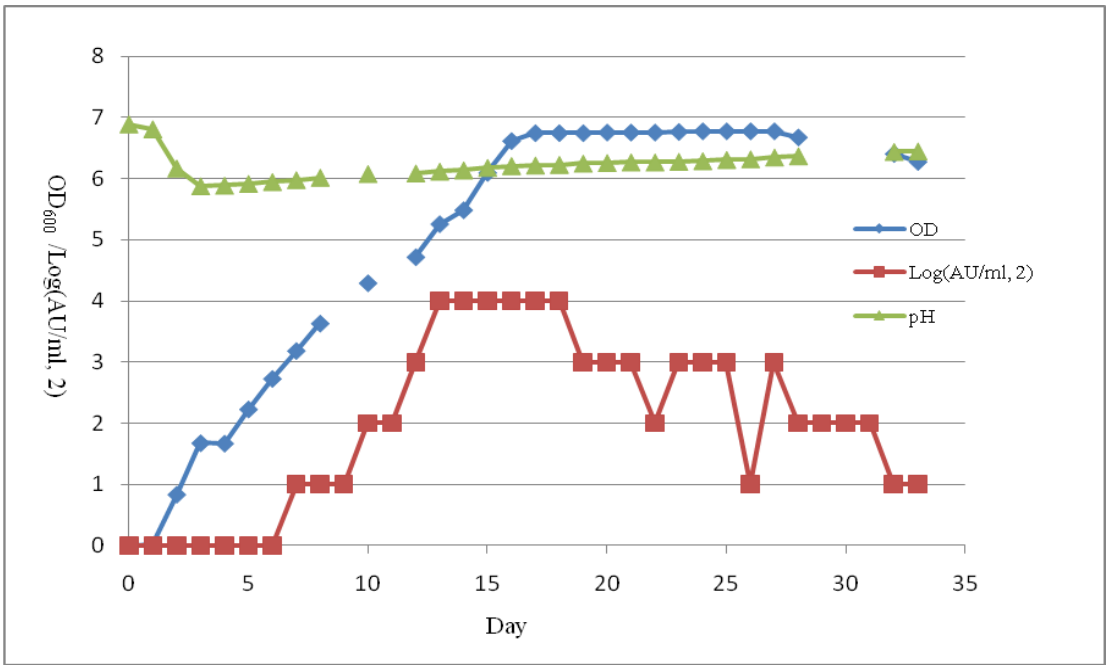

Figure 2.2 The OD, bioactivity, and pH of the first batch of *P. jensenii* cultivated in a bioreactor.

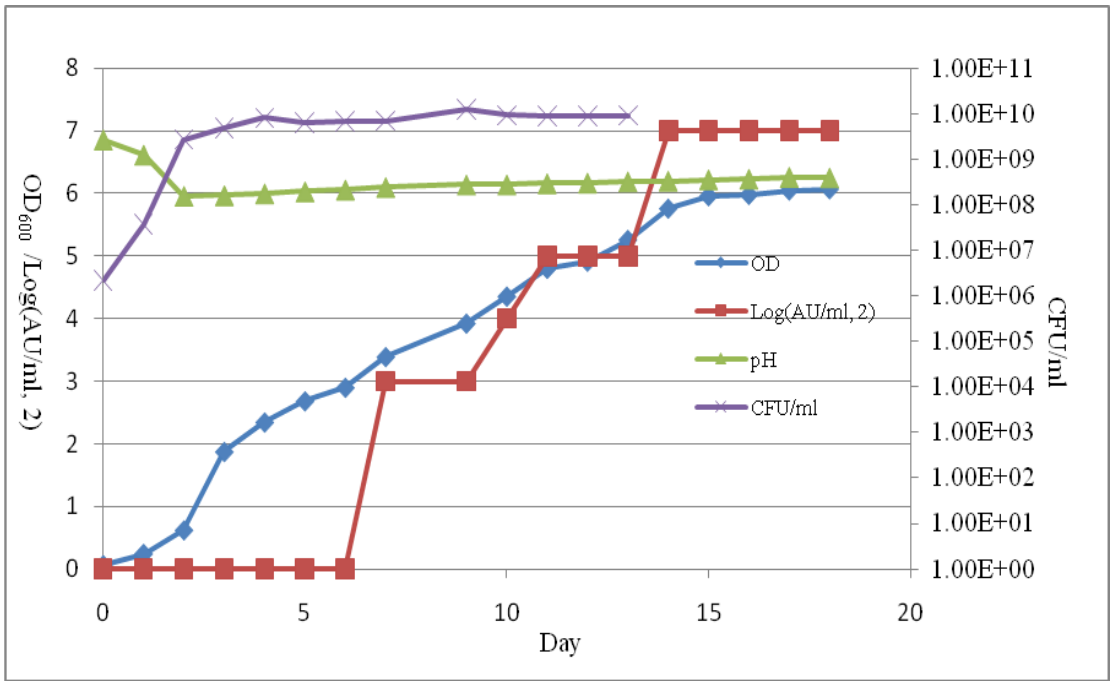

Figure 2.3 The OD, CFU, bioactivity, and pH of the second batch of *P. jensenii* cultivated in a bioreactor.

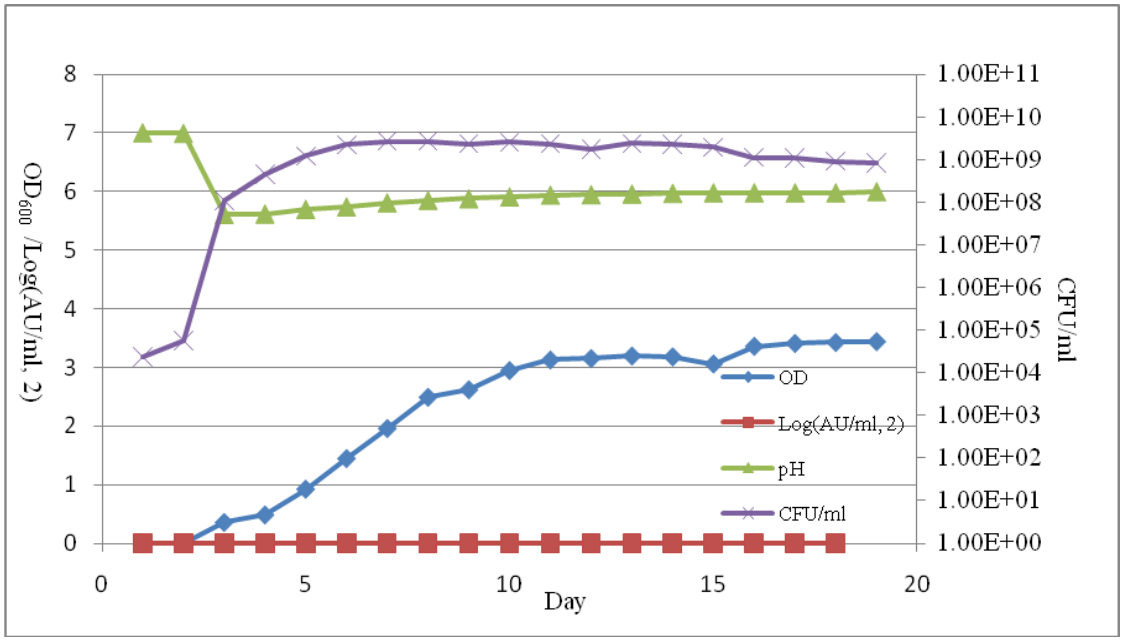

Figure 2.4 The OD, CFU, bioactivity, and pH of the fourth batch of *P. jensenii* cultivated in a bioreactor.

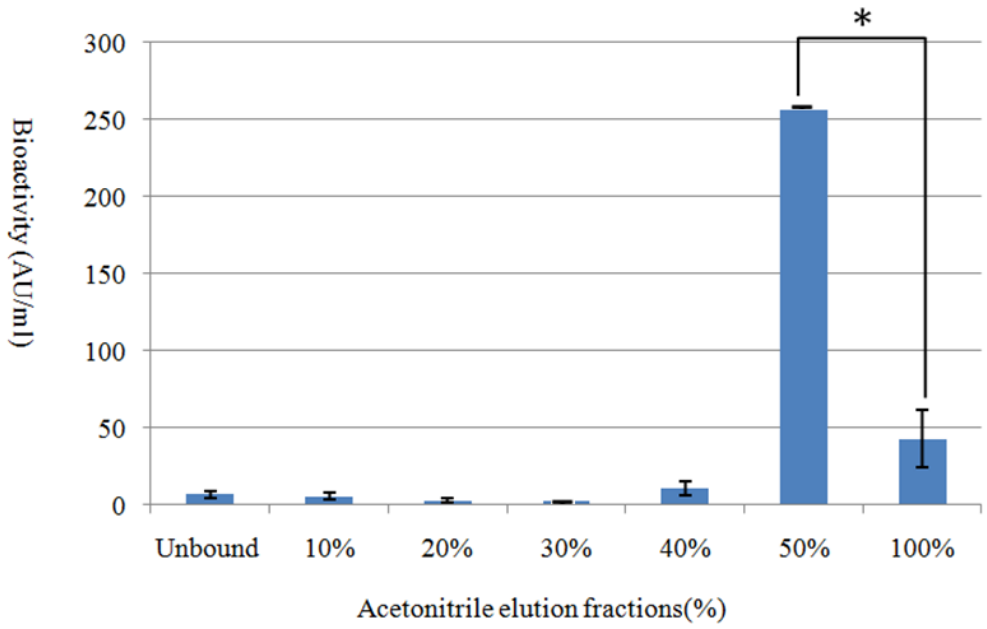

Figure 2.5 Bioactivity of each acetonitrile elution fraction from C18 resin silica column. Significant difference from other fractions (*$p<0.05$). Brackets indicate significant difference ($p<0.05$)

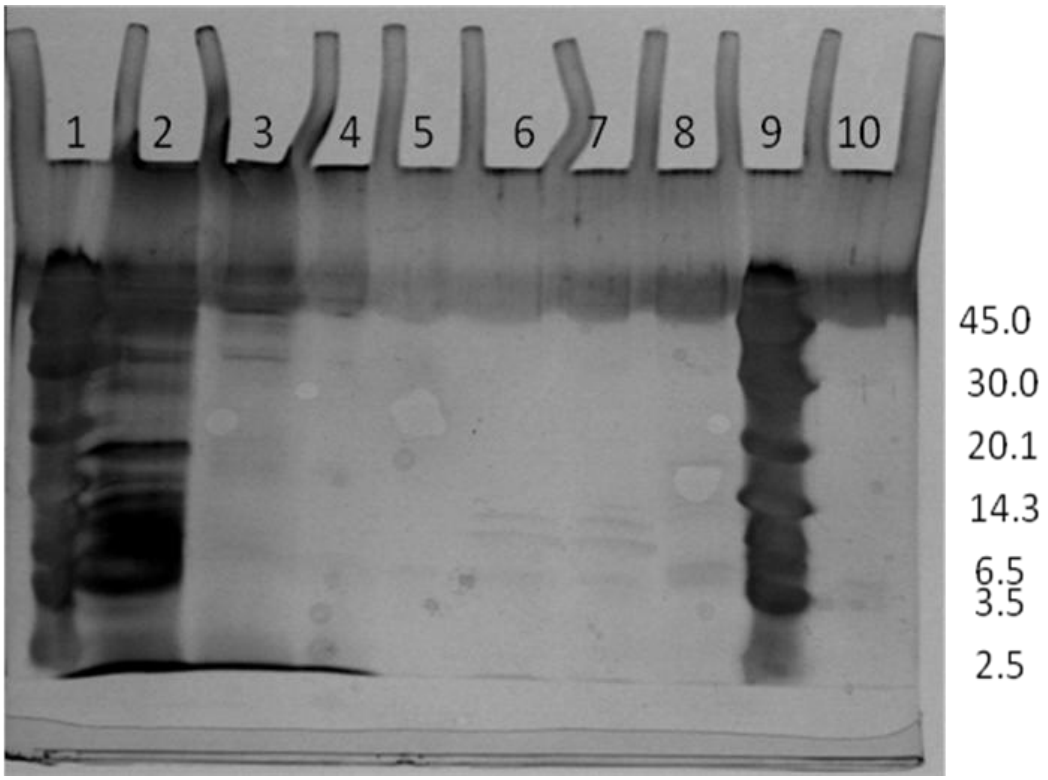

Figure 2.6. Different elution fractions from C18 resin silica column on SDS-PAGE with silver stain. Lane 1, marker; Lane 2, concentrated jenseniin P; Lane 3, unbound wash; Lane 4, 10 % acetonitrile elution; Lane 5, 20%; Lane 6, 30%; Lane 7, 40%; Lane 8, 50%; Lane 9, marker; Lane 10, 100%

Table 2.1 Purification result of jenseniin P

	Bioactivity (AU/ml)	Total Protein (mg/ml)	Specific Activity (AU/mg)	Purification Factor (Fold)
Supernatant of Day 18	128 ±0	6.61 ±0.75	19.55 ±2.10	1.00
Concentrated Supernatant	1877 ±418	12.86 ±2.30	152.82 ±52.15*	7.81
50% elution Fraction	256 ±0	0.69 ±0.05	372.23 ±24.88*	19.04

Significant difference from the specific activity of supernatant of day 18 ($p<0.001$), n=6.

CHAPTER THREE

CHARACTERIZATION OF FURTHER PURIFIED JENSENIIN P

3.1 Introduction

The activity of bacteriocin can be affected by different conditions. For example, temperature can affect the activity of some bacteriocins: at high temperature, the propionicin PLG-1 loses activity while jenseniin G remains stable(Lyon and Glatz 1991; Grinstead and Barefoot 1992). Some bacteriocins are pH-dependent because pH affects their net charge. For example, the lantibiotic and non-lanthionine-containing bacteriocins show higher activity at lower pH (pH 5 and below) than at physiological pH; their adsorption to the cell surface of Gram-positive bacteria, including the producing cells is pH-dependent, too(Jack, Tagg et al. 1995).

Determination of the molecular weight, mode of action, heat stability, and pH stability of jenseniin P, as well as the inflammatory response tests are the subjects of this present work.

3.2 Materials and Methods

3.2.1 Enzyme Treatment

Twenty-five µl each of 10mg/ml aqueous solution of trypsin, protease K,

pronase, protease XIV, lysozyme, and catalase were loaded onto sterilized disks of 7 mm in diameter on MRS agar plates. Twenty-five µl of jenseniin P (50% acetonitrile elution fraction) was loaded onto another disk next to the disk with enzyme on it. The distance between the two disks was approximately 1 mm. After 1 hour of incubation in 37 ℃, the plate was overlaid with *L. delbrueckii* in MRS soft agar and incubated in 37 ℃ for overnight. The clear zones around the disks were evaluated afterward.

3.2.2 Mode of Action

An overnight culture of *L. delbrueckii* was centrifuged and resuspended in 0.05M sodium phosphate buffer. OD_{600} of the suspension was adjusted to 1.0, which is equivalent to 1.1 x 10^7 CFU/ml. Five hundred µl of indicator strain and 500 µl of jenseniin P (50% acetonitrile elution fraction) were mixed and held in 37 ℃ for 60 min. At every 10 min interval, 100 µl samples were obtained from the mixture, diluted tenfold, and plated out onto MRS agar plates. The reduction in CFU/ml was recorded.

3.2.3 SDS-PAGE Analysis

Three identical 25 µl of jenseniin P from the 50% acetonitrile elution fraction were loaded onto three separate lanes of an 18% SDS-PAGE gel, and 10 µl of low-range rainbow molecular weight marker (Amersham biosciences, RPN 755)

was loaded into a separate lane. The SDS-PAGE gel ran at a constant voltage of 100V for 45 min. The gel was washed in 200 ml of 2.5% Trion X-100 for 1 h then in 200 ml of sterile distilled water for 4 h at room temperature(Miescher, Stierli et al. 2000). The gel was overlaid with 0.75% soft MRS agar inoculated with *L. delbrueckii* subsp. *lactis* ATCC 4797. The gel was incubated at 37 ℃ for overnight.

3.2.4 Heat Stability

Jenseniin P (50% acetonitrile elution fraction) was held in boiling water in eppendorf tubes for 90 min. At every 10 min interval, 100 µl of the sample were taken out and placed on ice. The activity of the sample was measured using the microtiter plate method.

3.2.5 pH Stability

Twenty µl of concentrated jenseniin P in 0.05 M sodium phosphate buffer (256 AU/ml) or sodium phosphate buffer controls were mixed with 300 µl each of standard solution with pH of 1, 2, 7, 12, and 13. HCl and NaOH were added into sterile distilled water to prepare the standard solution. After mixing, the resulting pH of the mixtures became 1.9, 4.2, 6.4, 7.0, and 12.8. The mixtures were then incubated at room temperature for 2 h. The activity of the sample after the treatment was measured using the microtiter plate method. Two-fold dilutions were made by using sodium phosphate buffer (pH 6.4).

3.3 Results and Discussion

3.3.1 Enzyme Treatment

In this present work, activity of jenseniin P was affected by trypsin, protease K, and pronase; activity of jenseniin P was not affected by protease XIV, lysozyme and catalase. If the enzyme did not inactivate jenseniin P, a round, clear inhibition zone around the disks loaded with jenseniin P was observed. Otherwise, one side of the inhibition zone was smaller than the other side (Fig 3.1). Trypsin cleaves proteins at the lysine or arginine residues. Protease K is a broad spectrum protease. Pronase can cause complete digestion of proteins.

3.3.2 Mode of Action of Jenseniin P

Most bacteriocins produced by LAB have a bactericidal mode of action(Ennahar, Sashihara et al. 2000). Treatment of *L. delbrueckii* with jenseniin P caused 99% reduction in the population in 60 min. This result indicated that jenseniin P has a bactericidal mode of action instead of bacteriostatic; the killing rate was calculated to be 2.38 log h^{-1} (Fig 3.2). To further determine whether the killing activity is bacteriolytic, electro microscopic imaged of jenseniin P treated cells can be analyzed. In addition, the release of DNA, if any, can be measured by using DNA gel electrophoresis, SYBR Green nucleic acid stain, or PCR.

3.3.3 Molecular Weight Determination

After running 45 min in SDS-PAGE gel, the gel was washed with Triton X-100 and distilled water to remove SDS and then overlaid with *L. delbrueckii*. A clear zone was formed because jenseniin P inhibited the growth of *L. delbrueckii*. The molecular weight was determined to be around 5 kDa. The estimated molecular weight of 5 kDa is based on comparing the migration distance of the center of the inhibition zone comparing with the markers using a longer (20cm) SDS-PAGE gel (data not shown). The shorter (10cm) SDS-PAGE gel was used to demonstrate the result of the bioassay (Fig3.3) due to the difficulty in photographing large gel. In previous study, the bioassay was interfered by the presence of SDS in the gel. In this present work, SDS was removed via washing with Triton X-100 as described in materials and methods section. As shown in Figure 3.5, the inhibition zone was pretty tight, which allowed us to accurately determine the molecular weight. Furthermore, the use of denaturing gel (SDS-PAGE) also allowed us to determine its true molecular weight, since jenseniin P either was stable to various concentrations of SDS(Ratnam 1997) or renatured after the Trion X-100 washing step. Although the use of native gels would allow us to determine the antimicrobial activity, molecular weight cannot be accurately determined with native gels; mobility in a native gel matrix depends on both protein charge and hydrodynamic size(Jack, Tagg et al. 1995).

3.3.4 Heat Stability

In the heat treatment test, the activity of jenseniin P decreased as time increased, but after treatment in boiling water for 90 minutes, the bioactivity of jenseniin P can still be detected with an activity of 10 AU/ml (Fig 3.4), approximately a 2-log reduction over 90 min. In SDS-PAGE analysis, after silver staining, the jenseniin P band became lighter and lighter from 0 min to 90 min (the band between 3.5 to 6.5 kDa) (Figure 3.5).

3.3.5 pH Stability

Under different pH conditions, jenseniin P exhibited similar activity, which indicated that the activity of jenseniin P was not affected by pH ranging from 1.9 to 12.8 (Table 3.1). Control groups with *L. delbrueckii* exposing to the same pH solutions did not show significant differences in viability, which indicated that the inhibitory effects observed in the experimental group were caused by jenseniin P, not by change in pH.

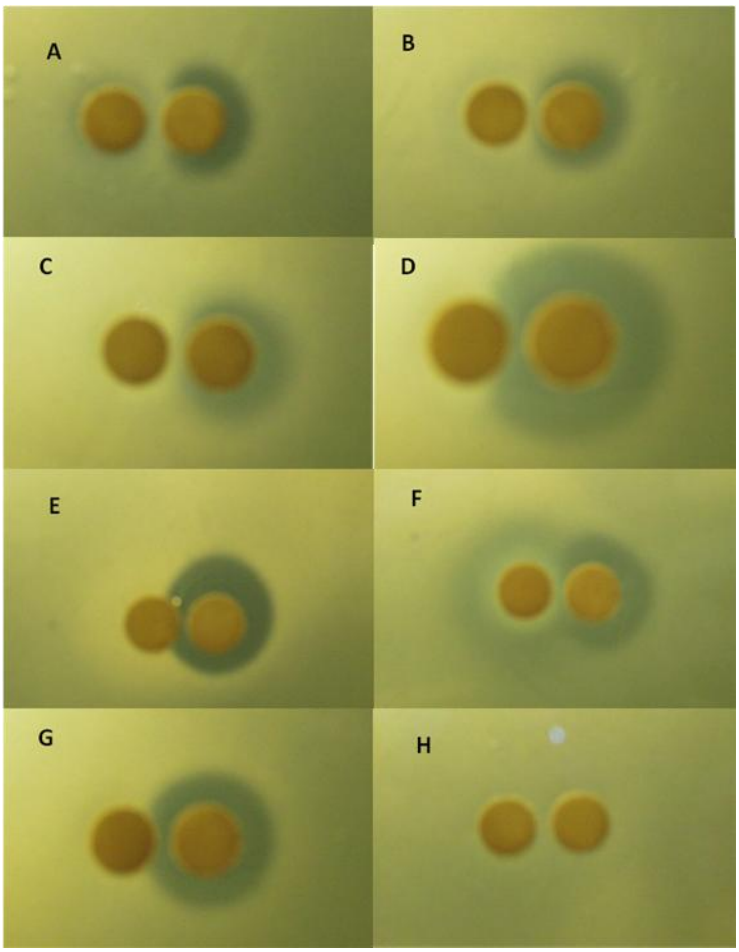

Figure 3.1. Effect of enzyme treatment on further purified jenseniin P (50 % acetonitrile elution fraction) Left disks: A. trypsin; B. protease K; C. pronase; D. protease XIV; E. catalase; F. lysozyme. G&H. MRS broth. Right disks: A-G. Further purified jenseniin P (50% acetonitrile elution fraction); H. 0.05 M sodium phosphate buffer.

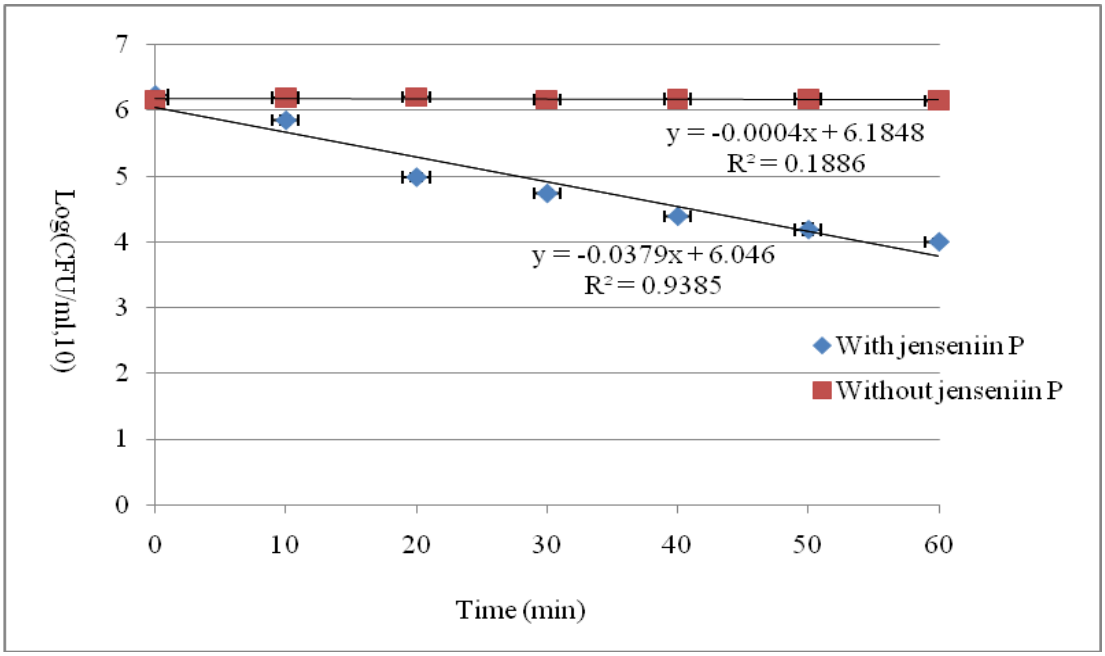

Figure 3.2 Killing rate of partially purified jenseniin P (50% acetonitrile elution fraction) on *L. delbrueckii,* n=3.

Figure 3.3 SDS-PAGE and overlay of further purified jenseniin P (50% acetonitrile elution fraction). Lane 1, 3, and 5 are identical further purified jenseniin P samples; Lane 10 is marker.

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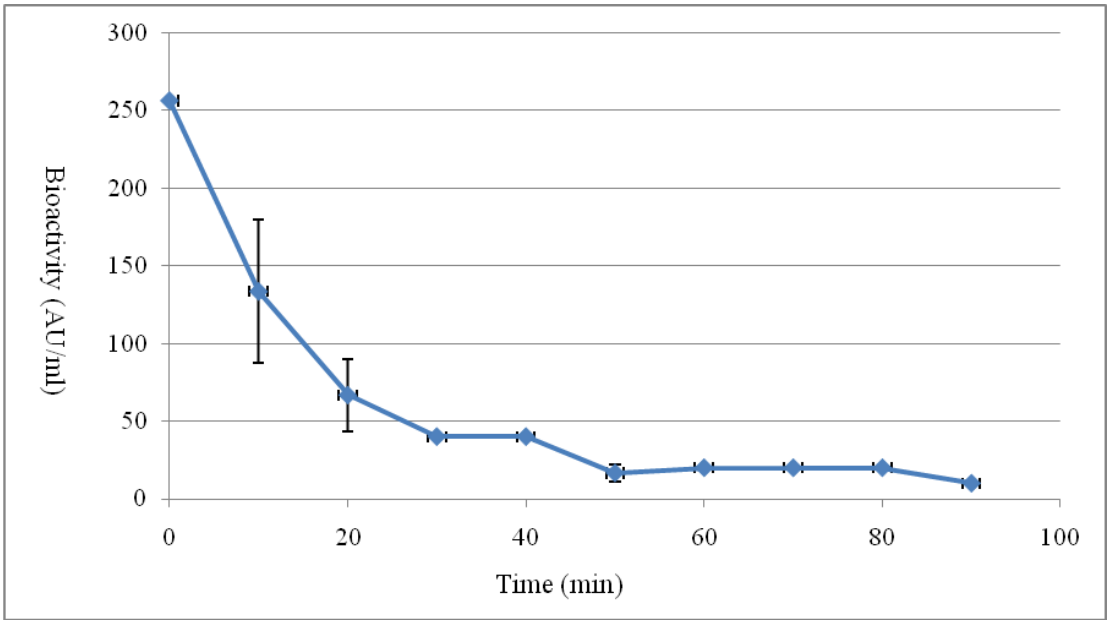

Figure 3.4 Heat treatment of partially purified jenseniin P (50% acetonitrile elution fraction), n=3.

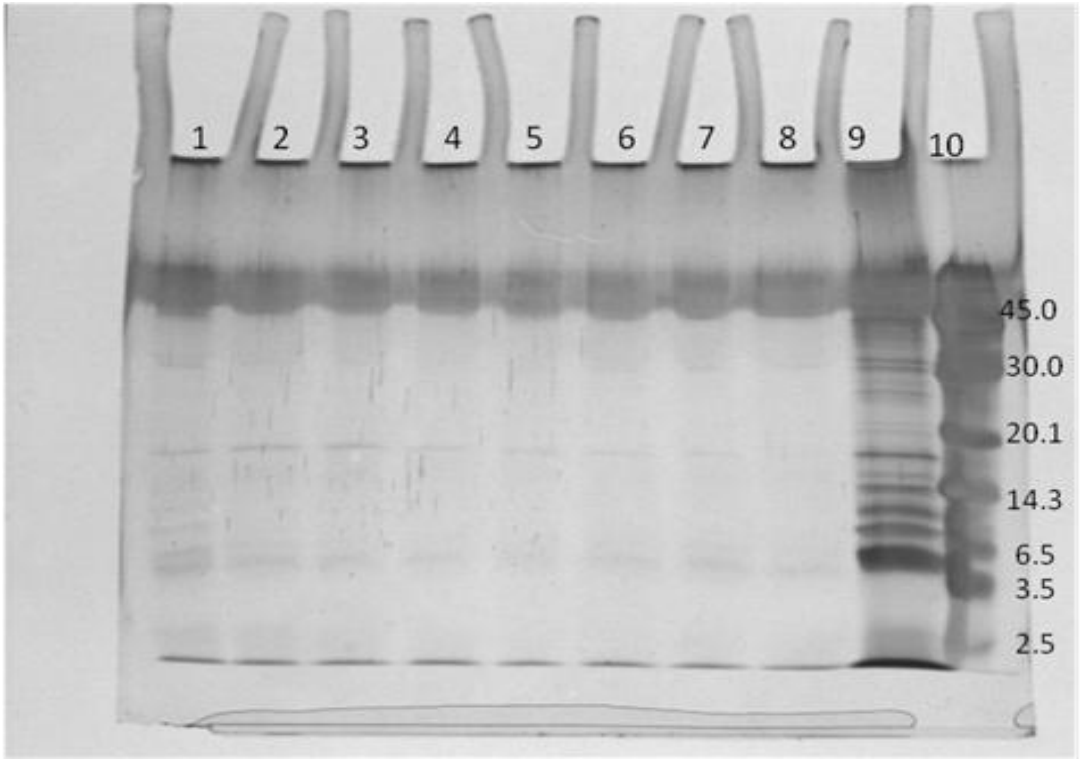

Figure 3.5 Effect of heat treatment on further purified jenseniin P (50% acetonitrile elution fraction)(Lanes 1-8) and concentrated jenseniin P. Lane1, 0 min; Lane 2, 10 min; Lane 3, 20 min; Lane 4, 30 min; Lane5, 40 min; Lane 6, 50 min; Lane 7, 70 min; Lane 8, 90 min; Lane 9, concentrated jenseniin P; Lane10, marker.

Table 3.1 Effect of pH on activity of jenseniin P

pH of standard solution	pH of diluted jenseniin P	Activity(AU/ml)
1	1.9	128 ±0
2	4.2	128 ±0
7	6.4	128 ±0
12	7.0	128 ±0
13	12.8	128 ±0

No significant difference between different samples.

CHAPTER FOUR

ANTI-ACNE AGENT PRODUCED BY *P. JENSENII* B1264

4.1 Introduction

In previous reports, antimicrobial activity of *P. jensenii* B1264 against *L. delbrueckii* ATCC 4797 and *P. acnes* 6919 was assumed to be due to one agent, jenseniin P. But according to Klaenhammer, 3%-99% of bacteria and archaea can produce more than one bacteriocin(Klaenhammer 1988). It is possible that the crude extract of *P. jensenii* B1264 contained more than one inhibitory agent. In this present work, it is determined that jenseniin P, which inhibits the growth of *L. delbrueckii,* is not the agent that inhibits the growth of *P. acnes* 6919.

4.2 Materials and Methods

4.2.1 Bacteriocin Activity Spot Assay Method

For evaluation of bacteriocin activity against *L. delbrueckii*, 10 ml of 1.5% agar was poured to form a base layer, and then overlaid with 5 ml soft MRS agar inoculated with overnight culture of *L. delbrueckii* in MRS broth (Final OD_{600} 0.02 after inoculation). Ten µl of 10%, 20%, 30%, 40%, 50%, 100% acetonitrile wash, and unbound fractions were spotted individually onto the top of the plate. The plate was

then incubated at 37 ℃ for 12 hours.

For evaluation of bacteriocin activity against *P. acnes*, 10 ml of 1.5 % agar were poured to form a base layer, and then overlaid with 5 ml soft NLA agar inoculated with 5 day *P. acnes* 6919 (Final OD_{600} 0.02 after inoculation). Ten µl of 10%, 20%, 30%, 40%, 50%, 100% acetonitrile wash, and unbound fractions were spotted onto the top of the plate. The plate was then incubated at 37 ℃ anaerobically for 5 days.

4.3 Results and Discussion

The 50% acetonitrile elution fraction inhibited the growth of *L. delbrueckii* but did not inhibit the growth of *P. acnes* 6919. The 30% acetonitrile elution fraction inhibited the growth of *P. acnes* 6919 but did not inhibit the growth of *L. delbrueckii* (Fig 4.1). Prince has already characterized the agent that inhibits the growth of *L. delbrueckii* as a bacteriocin, but the agent in the 50% elution that inhibited *P. acnes* 6919 had not been discovered previously. The agent in the supernatant that inhibited the growth of *P. acnes* 6919 could be a metabolite other than a bacteriocin. In order to prove that such agent is a bacteriocin, its protein nature needs to be confirmed, and other possibilities need to be excluded. If it is determined to be a bacteriocin in future work, *P. acnes* 6919 should be included as an indicator strain in subsequent purification and characterization processes. The results (Fig 4.1) were reproducible in triplicate in one batch of C18 resin silica column process.

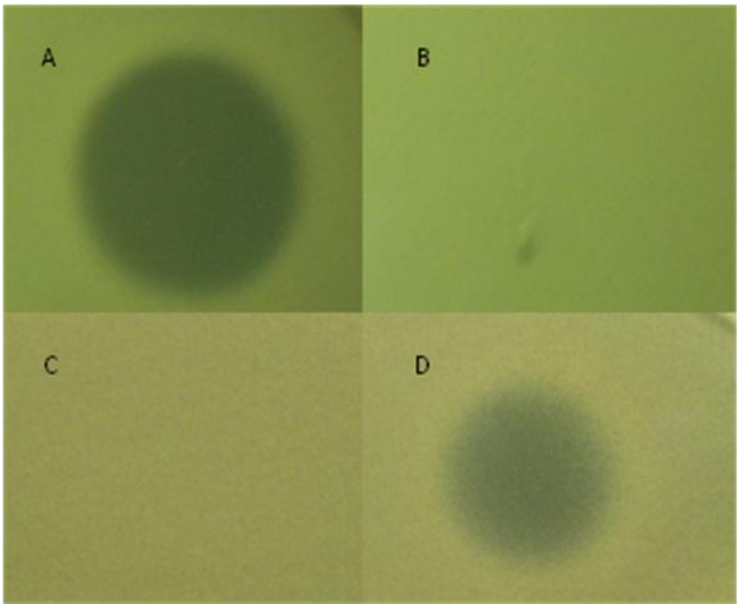

Figure 4.1. Spot-on-lawn assay of 50% acetonitrile eluent and 30% acetonitrile eluent. A. 50% acetonitrile eluent on *L. delbrueckii*; B. 30% acetonitrile eluent on *L. delbrueckii*; C. 50% acetonitrile eluent on *P. acnes* 6919; D. 30% acetonitrile eluent on *P. acnes* 6919.

CONCLUSIONS

The objective of Prince's work was to screen for bacteriocins produced by propionibacteria and she found that the supernatant of *P. jensenii* B1264 could inhibit 9 strains of propionibacteria and lactobacillus including itself. Ratnam partially purified the bacteriocin that could inhibit *L. delbrueckii* and characterized it. Ratnam further demonstrated that the partially purified jenseniin P was also inhibitory to *P. acne*s. The inhibitory activity to *L. delbrueckii* and to *P. acnes* 6919 was thought to be associated to the same bacteriocin and subsequent characterizations of jenseniin P were carried out using the *L. delbrueckii* as an indicator strain for it ease of cultivation and shorter generation time.

In this present work, we have demonstrated that the bacteriocin which inhibits the growth of *L. delbrueckii* is independent from the one that inhibits the growth of *P. acnes* 6919. Two independent approaches have been taken for further characterization of the antimicrobial agents produced by *P. jensenii* B1264. In one approach, we further purified the bacteriocin that could inhibit the growth of *L. delbrueckii* utilizing C18 resin silica column purification, a reverse phase chromatography. Additional purification methods could be utilized in future studies, such as ultra filtration, anion exchange chromatography, gel filtration, and HPLC or FPLC chromatography. The purified jenseniin P can be sent for

N-terminal sequencing. Degenerative primers based on the amino acid sequences can then be synthesized. The gene cluster can be amplified by PCR and the ORFs in the gene cluster can be analyzed and compared with known bacteriocins.

To localize the DNA region(s) that encodes for jenseniin P gene clusters, transposon mutagenesis on the whole genome of *P. jensenii* B1264 could be utilized. If bacteriocin defective mutants are generated, PCR could be utilized to amplify the insertion region of the transposon and its flanking sequence. After the DNA sequence is determined, the amino acid sequence can then be determined. The difficulty of this method is that, to-date, no transposon mutagenesis have been conducted with propionibacteria and the only sequenced genome in this genus is *P. acnes*.

In this molecular approach, one of the difficulties met by previous colleague was that the cells of *P. jensenii* B1264 was difficult to lyse, because the unique structure of its cell wall. We successfully lysed the cells by using the combination of 10mg/ml lysozyme and 25 unit/ml mutanolysin, which was the same method for lysing of *P. jensenii* DF1 as reported by Miescher(Miescher, Stierli et al. 2000). After lysing the cells, we tried to extract plasmid using Plasmid Mini Kit (QIAGEN, CA, Cat # 12123), which is designed for extraction of plasmid with a size range between 10bp and 10kb. However, no plasmid was observed on DNA agarose gel. The possible reasons might be that there was no or low copy number plasmids present in the cells, or the size of the plasmids exceeds the capability of

the plasmid extraction kit. To validate the precious statement, pLME106 containing *P. jensenii* DF1 strain can be used for the extraction of plasmid. If pLME106 can be extracted using this kit, then my statement is true. Otherwise, a more appropriate plasmid extraction method needs to be developed.

In this present work, the results support that jenseniin P is not the agent that inhibits the growth of *P. acnes* 6919. If this agent is proved to be a bacteriocin in the future, it will support the observations that most bacteria and achaea can produced more than one bacteriocins(Klaenhammer 1988).

In conclusion, we further purified and characterized jenseniin P, mainly via C18 resin silica column fractionation. Jenseniin P was found to be a small bacteriocin (3.5-6kDa) with a bactericidal mode of action. In addition, it was determined to be pH stable and heat stable, because it retains its bactericidal activity under various pH and after being treated in boiling water for 90 min. Another important discovery in this research is that P. jensenii B1264 might produce more than one bacteriocin. Jenseniin P and the agent that inhibit P. acnes 6919 need to be further studied in the future.

APPENDIX

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End page	582
Type of Use	Dissertation/Thesis
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Number of figures/tables	1
Order reference number	
Title of your thesis / dissertation	FURTHER PURIFICATION AND CHARACTERIZATION OF JENSENIIN P, A BACTERIOCIN PRODUCED BY PROPIONIBACTERIUM JENSENII B1264
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REFERENCES

Barefoot, S. F. and T. R. Klaenhammer (1984). "Purification and characterization of the Lactobacillus acidophilus bacteriocin lactacin B." Antimicrob Agents Chemother **26**(3): 328-334.

Bergey, D. H. and J. G. Holt (1994). Bergey's manual of determinative bacteriology. Baltimore, Md., Williams & Wilkins.

Bershad, S. V. (2001). "The modern age of acne therapy: a review of current treatment options." Mt Sinai J Med **68**(4-5): 279-286.

Brede, D. A., T. Faye, et al. (2004). "Molecular and genetic characterization of propionicin F, a bacteriocin from Propionibacterium freudenreichii." Appl Environ Microbiol **70**(12): 7303-7310.

Burton, J. L., W. J. Cunliffe, et al. (1971). "The prevalence of acne vulgaris in adolescence." Br J Dermatol **85**(2): 119-126.

Cammue, B. P., M. F. De Bolle, et al. (1992). "Isolation and characterization of a novel class of plant antimicrobial peptides form Mirabilis jalapa L. seeds." J Biol Chem **267**(4): 2228-2233.

Cole, A. M., P. Weis, et al. (1997). "Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder." J Biol Chem **272**(18): 12008-12013.

Cotter, P. D., C. Hill, et al. (2005). "Bacteriocins: developing innate immunity for food." Nat Rev Microbiol **3**(10): 777-788.

Cunliffe, W. J. and D. J. Gould (1979). "Prevalence of facial acne vulgaris in late adolescence and in adults." Br Med J **1**(6171): 1109-1110.

Dessinioti, C. and A. D. Katsambas (2010). "The role of Propionibacterium acnes in acne pathogenesis: facts and controversies." Clinics in Dermatology **28**(1): 2-7.

Drider, D., G. Fimland, et al. (2006). "The continuing story of class IIa bacteriocins." Microbiol Mol Biol Rev **70**(2): 564-582.

Dutton, C. J. (2002). Peptide antibiotics : discovery, modes of action, and applications. New York, Marcel Dekker.

Ennahar, S., T. Sashihara, et al. (2000). "Class IIa bacteriocins: biosynthesis, structure and activity." FEMS Microbiol Rev **24**(1): 85-106.

Ennahar, S., K. Sonomoto, et al. (1999). "Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation." J Biosci Bioeng **87**(6): 705-716.

Faye, T., D. A. Brede, et al. (2002). "An antimicrobial peptide is produced by extracellular processing of a protein from Propionibacterium jensenii." J Bacteriol **184**(13): 3649-3656.

Faye, T., T. Langsrud, et al. (2000). "Biochemical and genetic characterization of propionicin T1, a new bacteriocin from Propionibacterium thoenii." Appl Environ Microbiol **66**(10): 4230-4236.

Froyshov, O. (1977). "The production of bacitracin synthetase by Bacillus licheniformis ATCC 10716." FEBS Lett **81**(2): 315-318.

Goodman, G. J. (1999). "Acne and acne scarring: why should we treat?" The Medical Journal of Australia **171**: 62-63.

Gratia, A. (1925). "Sur un remarquable exemple d'antagonisme entre deux souches de colibacille C. R. Seances." Soc. Biol. Fil **93**: 1040–1041.

Grinstead, D. A. and S. F. Barefoot (1992). "Jenseniin G, a heat-stable bacteriocin produced by Propionibacterium jensenii P126." Appl Environ Microbiol **58**(1): 215-220.

Jack, R. W., J. R. Tagg, et al. (1995). "Bacteriocins of gram-positive bacteria." Microbiol Rev **59**(2): 171-200.

Kang, B. S., J. G. Seo, et al. (2009). "Antimicrobial activity of enterocins from Enterococcus faecalis SL-5 against Propionibacterium acnes, the causative agent in acne vulgaris, and its therapeutic effect." J Microbiol **47**(1): 101-109.

Klaenhammer, T. R. (1988). "Bacteriocins of lactic acid bacteria." Biochimie **70**(3): 337-349.

Klaenhammer, T. R. (1993). "Genetics of bacteriocins produced by lactic acid bacteria." FEMS Microbiology Reviews **12**(1-3): 39-85.

Lehrer, R. I., A. K. Lichtenstein, et al. (1993). "Defensins: antimicrobial and cytotoxic peptides of mammalian cells." Annu Rev Immunol **11**: 105-128.

Lyon, W. J. and B. A. Glatz (1991). "Partial Purification and Characterization of a Bacteriocin Produced by Propionibacterium thoenii." Appl. Environ. Microbiol. **57**(3): 701-706.

Miescher, S., M. P. Stierli, et al. (2000). "Propionicin SM1, a bacteriocin from Propionibacterium jensenii DF1: isolation and characterization of the protein and its gene." Syst Appl Microbiol **23**(2): 174-184.

Nes, I. F., D. B. Diep, et al. (1996). "Biosynthesis of bacteriocins in lactic acid bacteria." Antonie van Leeuwenhoek **70**(2-4): 113-128.

Nettles, C. G. and S. F. Barefoot (1993). "Biochemical and genetic characteristics of bacteriocins of food-associated lactic acid bacteria." J. Food Prot **56**: 338-356.

O'Sullivan, L., R. P. Ross, et al. "Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality." Biochimie **84**(5-6): 593-604.

Prince, L. D. (1993). "Detection and partial characterization of a bacteriocin, jenseniin P, from Propionibacterium jensenii P1264." M.S.Thesis. : Clemson University, Clemson, SC.

Ramanathan, S., C. Wolynec, et al. (1968). "Antiviral principles of Propionibacteria-isolation and activity of propionins B and C." Proc Soc Exp Biol Med **129**(1): 73-77.

Ratnam, P. (1997). "Partial purification and characterization of the anti-acne bacteriocin jenseniin P." PhD Dissertation. (Clemson University, Clemson, SC).

Steiner, H., D. Hultmark, et al. (1981). "Sequence and specificity of two antibacterial proteins involved in insect immunity." Nature **292**(5820): 246-248.

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Zasloff, M. (1987). "Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor." Proc Natl Acad Sci U S A **84**(15): 5449-5453.