รายงานวิจัยฉบับสมบูรณ์

โครงการ การผลิตสารชีวสังเคราะห์จาก *Lactobacillus paracasei* HL32 เพื่อควบคุมเชื้อก่อโรคปริทันต์

The production of biosynthetic substances from *Lactobacillus paracasei* HL32 for bacterial control in periodontal diseases

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กรกฎาคม 2552

สัญญาเลขที่ RMU 4980041

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและสำนักงานคณะกรรมการการอุดมศึกษา
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

สารบัญ

กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับทุนอุดหนุนจากสำนักงานคณะกรรมการการอุดมศึกษา (สกอ.)และสำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ตามโครงการทุนพัฒนานักวิจัยประจำปี 2549 สัญญาเลขที่ RMU 4980041 และโดยการสนับสนุนด้านเครื่องมือและห้องปฏิบัติการจากสถานวิจัยความเป็นเลิศระบบนำส่งยา และคณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์

บทคัดย่อ

รหัสโครงการ RMU 4980041

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วัตถุประสงค์

1) สร้างนวัตกรรมในการค้นพบสารต้านจุลชีพกลุ่มเปปไทด์ที่ออกฤทธิ์เฉพาะเจาะจงต่อเชื้อก่อโรคปริทันต์
2) เพื่อช่วยเพิ่มประสิทธิภาพในการรักษาโรคปริทันต์อักเสบเรื้อรังชนิดรุนแรงและชนิดรุกราน
3) สามารถทราบถึงกลไกการฆ่าเชื้อของสารเปปไทด์หรือโปรตีนต่อเชื้อก่อโรคปริทันต์
4) ได้สารต้านจุลชีพเปปไทด์ชนิดใหม่
5) ทราบกลไกการออกฤทธิ์และองค์ประกอบสำคัญที่ทำให้เปปไทด์มีฤทธิ์เพื่อใช้ในการออกแบบยา
6) สร้างแนวทางการผลิตยาโดยอาศัยชีวสังเคราะห์จากแบคทีเรีย

วิธีทดลอง

1. การเตรียมและเพาะเลี้ยงเชื้อ *L. paracasei* HL32 ในระดับ pilot scale โดยอาศัย bioreactor เมื่อได้ supernatant จะทำให้บริสุทธิ์มากขึ้นโดยอาศัย tangential flow ultra-filtration ขั้นตอนนี้ต้องทำการหาสภาวะที่เหมาะสมในการทำให้บริสุทธิ์ที่สามารถแยกสารอื่นๆ ที่ไม่ต้องการออกให้เหลือเฉพาะ bacteriocin น้ำหนักโมเลกุล 56 kDa ก่อนนำไปทดสอบฤทธิ์ในการฆ่าเชื้อแบคทีเรีย ตรวจสอบเอกลักษณ์โดยใช้ mass spectrometry, TLC, SDS-PAGE และอื่นๆ

2. การทดสอบผลการยับยั้งและการฆ่าเชื้อแบคทีเรีย แบคทีเรียที่ใช้ทดสอบ ได้แก่ ตัวแทนแบคทีเรียก่อโรคปริทันต์ 4 ชนิด สายพันธุ์ *P. gingivalis* W 50 และ ATCC 33277 *Prevotella intermedia* (ATCC 25611) และ *Tannerella forsythensis* (ATCC 43037) ตัวแทนแบคทีเรียประจำถิ่นในช่องปาก 2 ชนิด *Streptococcus salivarius* (25975) และ *Streptococcus sanguis* (ATCC 10556) ตัวแทนแบคทีเรียแกรมบวก *S. aureus* ATCC 25923 และ ATCC 29213 ตัวแทนแบคทีเรียแกรมลบ *E. coli* ATCC 25922

3. ศึกษากลไกการออกฤทธิ์ต้านเชื้อก่อโรคปริทันต์
ในการศึกษาการเปลี่ยนแปลงรูปร่างของผนังเซลล์แบคทีเรีย นำ suspension cell 1 มิลลิลิตร ที่ได้จากการศึกษาการฆ่าแบคทีเรีย มากรองผ่านเซลลูโลสเมมเบรน ขนาดเส้นผ่านศูนย์กลาง 0.22 ไมครอน โดยใช้ 2.5% glutaraldehyde ในฟอสเฟตบัฟเฟอซาไลน์เป็นตัวตรึง จากนั้นนำไปเคลือบทองแล้วส่องดูด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราด

4. การศึกษากลไกการฆ่าเชื้อระดับโมเลกุลโดยการตกผลึกสารบริสุทธิ์ที่ได้จากชีวสังเคราะห์เพื่อนำมาศึกษา X-ray crystallography เพื่อศึกษาโครงสร้างในระดับโมเลกุล

5. ตรวจสอบโมเลกุลของสารชีวสังเคราะห์ที่ส่งผลต่อการออกฤทธิ์ฆ่าเชื้อ ศึกษาฤทธิ์ฆ่าเชื้อของ bacteriocin ขณะที่ยังอยู่ในรูป glycosylated protein และขณะที่ย่อยคาร์โบไฮเดรต ออกไปแล้วและเมื่อทำการย่อยให้ได้สายเปปไทด์ที่สั้นลง ฤทธิ์ของสารเปปไทด์เป็นอย่างไร ตรวจสอบโมเลกุลโดยเทคนิค MALDI-TOF MS ประกอบกับ LC-MS-MS เพื่อหาลำดับกรดะมิโนและน้ำหนักโมเลกุล

6. การทดสอบความคงตัวของสารชีวสังเคราะห์ที่ pH และอุณหภูมิต่างๆ รวมทั้งในสภาวะที่อยู่ในน้ำลายและน้ำเหลืองเหงือก โดยการวิเคราะห์หาปริมาณสารชีวสังเคราะห์ทางเคมีและจุลชีววิทยา

7. การทดสอบความเป็นพิษของเซลล์เอ็นยึดปริทันต์ เซลล์ไฟโบรบลาสต์จากเนื้อเยื่อเหงือกและเซลล์เยื่อบุ และวิเคราะห์ความเป็นพิษต่อเม็ดเลือดแดง

ผลการทดลอง

โปรตีนจากการชีวสังเคราะห์ของ *Lactobacillus paracasei* HL32 ได้ถูกผลิตออกมา และทำให้บริสุทธิ์ เมื่อนำมาทดสอบคุณสมบัติทุกด้านพบว่าโปรตีนมีฤทธิ์ทางชีวภาพในการทำลายเชื้อก่อโรคปริทันต์ *P.gingivalis* ทั้งสองสายพันธุ์ และเมื่อทำให้บริสุทธิ์และตรวจสอบน้ำหนักโมเลกุล ลำดับกรดอะมิโน พบว่ามีความเป็นไกลโคโปรตีน เมื่อตัดน้ำตาลออกจะเหลือเฉพาะเปปไทด์ เมื่อทดสอบฤทธิ์ทางชีวภาพอีกครั้งหลังย่อยด้วยเอนไซม์อะไมโลกลูโคซิเดส พบว่าฤทธิ์การฆ่าเชื้อก่อโรคยังคงมีอยู่ เมื่อทดสอบความเป็นพิษต่อเซลล์เนื้อเยื่อเหงือกและเอ็นยึดปริทันต์พบว่าสารเปปไทด์ไม่มีพิษที่ระดับความเข้มข้น 16 mcM และเมื่อทดสอบความคงตัวต่อความร้อน พบว่าเปปไทด์ค่อนข้างคงตัวต่อความร้อน เมื่อทดสอบความคงตัวต่อน้ำลายพบว่าโปรตีนชีวสังเคราะห์มีฤทธิ์ทางชีวภาพ เมื่อนำมาศึกษากลไกการทำลายเชื้อก่อโรคปริทันต์ทั้งสองชนิด พบว่าโปรตีนจะทำลายเชื้อได้ดีทั้งสองกรณี

เมื่อนำโปรตีนมาศึกษารายละเอียดทางสัณฐาน ที่เกิดการทำลายเชื้อแบคทีเรีย พบว่าทั้ง SEM และ TEM ยืนยันผลในทำนองเดียวกัน คือ เชื้อถูกทำลายบริเวณผนังเซลล์ที่ส่วนปลาย ทำให้เกิดการแตกระเบิดที่ผนังทั้งสองด้านของเซลล์แบคทีเรีย ซึ่งเข้าใจว่าเกิดขณะมีการแบ่งตัวของเชื้อ เมื่อศึกษาภายในนิวเคลียสและด้านนอกเซลล์ พบว่า vesicle ของแบคทีเรียหายไป กลไกการถูกทำลายของเชื้อสายพันธ์ *P. gingivalis* w50 กับ *P. gingivalis* ATCC 33277 ด้วยโปรตีนชีวสังเคราะห์แตกต่างกันเล็กน้อย เมื่อนำเปปไทด์ที่ได้ทั้งสายมาหาจุดที่จะเกิดการทำลายเชื้อก่อโรคปริทันต์ โดยตัดด้วยเอนไซม์เปปซินและทดสอบฤทธิ์และสังเคราะห์เปปไทด์สายสั้นออกมา 9 สาย พบว่า fragment ทั้ง 9 สาย เมื่อนำมาทดสอบฤทธิ์ต่อเชื้อก่อโรค พบว่ามีเพียงเปปไทด์ 2 ชนิดเท่านั้นที่มีฤทธิ์ต่อเชื้อก่อโรคปริทันต์ ซึ่งตรวจสอบลำดับกรดอะมิโนของเปป

สรุปและวิจารณ์ผลการทดลอง

ในงานวิจัยได้สารชีวสังเคราะห์ของ *Lactobacillus paracasei* HL32 บริสุทธิ์ และมีฤทธิ์ต้านจุลชีพกับเชื้อก่อโรคปริทันต์ และทราบถึงโครงสร้างส่วนที่มีผลต่อการออกฤทธิ์ต้านจุลชีพดังกล่าวอย่างจำเพาะเจาะจง โดยออกฤทธิ์ทำลายผนังเซลล์ ทำให้เซลล์ไม่สามารถแบ่งตัวได้สำเร็จและมีการรั่วไหลของอิออนทำให้เซลล์ตาย และสามารถสังเคราะห์เปบไทด์ทางเคมีในส่วนที่ออกฤทธิ์ได้เป็นผลสำเร็จ

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

นักวิจัยต้องเข้าใจในกลไกการทำลายเชื้อ รวมถึงการออกแบบโมเลกุลให้มีความเป็นพิษต่อเม็ดเลือดแดงหรือเซลล์ที่เกี่ยวข้องในร่างกายต่ำ ในอนาคตการทำนายโครงสร้างที่มีโอกาสเป็นไปได้ในสิ่งแวดล้อมของร่างกายจะช่วยให้เข้าใจกลไกการออกฤทธิ์ได้มากขึ้น

คำหลัก

Lactobacillus paracasei, Biosynthesis, Periodontal diseases, bacteriocin, antimicrobial, biotechnology, lactic acid bacteria, mechanism of action

Abstract

Project Code: RMU 4980041

Project Title: The production of biosynthetic substances from *Lactobacillus paracasei* HL32 for bacterial control in periodontal diseases

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Project Period: 20 July 2006 - 20 July 2009

Objectives: Purification, identification and characterization of bacteriocin produced by *Lactobacillus paracasei* HL32. It has been shown to have activity against *Porphyromonas sp.* The mechanism of action of the bacteriocin was investigated and indentified for crucial element for anti-periodontitis.

Methodology: The purification of bacteriocin consisting of gel exclusion followed by anion exchange chromatography produced a single band upon an electrophoresis gel with a molecular weight corresponding to 56 kDa. The isolated protein contained 171 amino acids and the first 151 were sequenced. The bacteriocin contained a high percentage of cationic amino acids near the N-terminus, hydrophobic amino acids in the central region (Leu, Ile, Val, Phe, Trp and Gly) and hydrophilic residues (Ser, Asn and Gln) at the C-terminus. This structure did not match with that of previously reported bacteriocins. The antimicrobial activity of the bacteriocin was determined against some pathogens and normal microbiota (*P. gingivalis, P. intermedia, T. forsythensis, S. salivarius* and *S. sanguinis*) found in saliva and crevicular fluid. The chemical synthesis of peptide fragment from the bacteriocin was carried out. The mechanism of action was investigated using SEM and TEM for morphology and internal structure of pathogenic bacteria.

Results and discussion: The bacteriocin was found to inhibit *P. gingivalis* at the minimum bactericidal concentration (MBC) of 0.14 mM, but was found not to inhibit the other oral microorganisms. The bacteriocin was found from transmission electron microscopy studies to cause pore formation in the cytoplasmic membranes of *P. gingivalis* at the pole and induce potassium efflux. Bacteriocin concentrations of 2-4 times of MBC were shown to induce hemolysis. The bacteriocin was heat-stable, surviving at 110 ^{o}C under pressure and possessed

activity over a pH range of 6.8-8.5. Only a small reduction of activity was found to occur after incubation in biological fluids (saliva and crevicular fluid). When the bacteriocin was cleaved to obtain only polypeptide following by digestion with pepsin. Some peptide fragments (2 from 9 segments) were active against *P. gingivalis*. The two active fragments have not been reported in the literature or been patented. Therefore, we expect to patent the active peptide sequence. Active peptide was successfully synthesized and purified. The structure was confirmed by the mass analysis. The active peptide was crystallized to obtain single crystal suitable for X-ray crystallography (have not obtained results yet). The computer simulation of active peptide revealed the beta sheet and alpha helix structure were obtained with lowest energy. The antibacterial activity was confirmed again with the damage of bacterial cell wall during the cell division.

Conclusion: A novel bacteriocin has been identified and has selective activity against *Porphyromonas* sp. associated with periodontal disease. The purified active peptide was obtained from chemical synthesis. The antibacterial activity was identified and the damage to bacterial cell was evidenced.

Suggestions: The findings of this work gained the knowledge of specific antibacterial activity of bacteriocin against *Porphyromonas gingivalis*.

Keywords:

Lactobacillus paracasei, Biosynthesis, Periodontal diseases, bacteriocin, antimicrobial, biotechnology, lactic acid bacteria, mechanism of action

INTRODUCTION

Lactobacillus is an ubiquitous bacteria in nature and has a long history of safe use in food products and in healthcare (Klaenhammer 1993; Holzapfel et al. 1998). In humans, lactobacilli are found in the mouth, lower intestine and vagina (Holzapfel et al. 1998). Over the past decades, there has been an increase in the use of lactobacillus as a live microorganism in certain 'pro- or pre-biotic' food items for promoting or supporting a balance of indigenous microflora. Claims have been made that such 'probiotics' may prevent certain infectious diseases of the gastrointestinal tract, as well as providing a potential source of antimicrobial agents, such as bacteriocins (Holzapfel et al. 1998; Kaewsrichan et al. 2004). However, the efficacy and mechanisms of such protective effects are not entirely understood (Montville, Winkowski and Ludescher 1992; Holzapfel et al. 1998; Hancock 2001; Duche 2002).

In earlier studies, the antimicrobial action of bacteriocins has been found to be selective, with activity found against Gram-positive organisms and species closely related to that of the bacteriocin producer (Schillinger and Lucke 1989; Daeschel, McKenney and McDonld 1990; Sobrino et al. 1991; Klaenhammer 1993). Later work presented evidence to show that bacteriocins also had inhibitory activity towards Gram-negative bacteria (Klaenhammer 1993; Suma, Misra and Varadaraj 1998). It is well known that bacteriocins can be found from both Gram-positive and Gram-negative bacteria. However, bacteriocins from Gram-negative bacteria were found to be relatively active against Gram-negative bacteria (Riley and Wertz 2002). It is generally accepted that bacteriocins act by targeting bacterial cytoplasmic membranes (Montville, Winkowski and Ludescher 1992; Klaenhammer 1993; Duche 2002). The composite amino acid residues of the bacteriocins confer a cationic amphiphilic structure, and such a characteristic is likely to contribute to their mechanisms of action (Zasloff 2002). Some have a composite structure of protein, lipid, and/or carbohydrate moieties (Montville, Winkowski and Ludescher 1992).

Most studies to date have focused on the purification, amino acid sequencing and description of the genetic determinants of the bacteriocin, and only a few studies examined the mechanism of action in detail. Mechanisms that have been proposed to account for the activity of bacteriocins include the possible formation of a barrel/stave poration complex, the destabilization of the bacterial membrane as a consequence of transient pore formation, and the depletion of the

proton-motive force, which can be a primary or secondary event (Montville, Winkowski and Ludescher 1992; Duche 2002; Hancock 2002).

Lactobacillus plantarum, L. rhamnosus, L. salivarius, L. casei, L. fermentum, L. acidophilus, L. oris, L. paracasei, L. brevis, L. buchneri, L. delbrueckii, L. jensenii, L. gasseri and *L. agilis* are the most common species found within the human oral cavity. The presence of such oral lactobacilli is correlated with good oral health, evidenced by the fact that most of these species have been shown to have the ability to inhibit the growth of both periodontal and caries-related pathogens *in vitro*. The prominent species i.e., *L. paracasei, L. plantarum, L. rhamnosus* and *L. salivarius*, have been shown to reduce the growth of periodontal pathogens (Sookkhee, Chulasiri and Prachyabrued 2001; Koll-Klais et al. 2005).

The secretion of bacteriocins has been proposed to occur as part of a survival strategy by organisms in the oral microbial population (Fujimura and Nakamura 1979; Nakamura et al. 1981). Moreover in recent years the prevalence of antibiotic-resistant strains of periodontal pathogens has increased providing a greater impetus to develop selective means of controlling oral pathogenic microorganisms (Walker 1996; Sanai et al. 2002; Chan and Chan 2003). The use of a bacteriocin from *Prevotella nigrescens* has been demonstrated to provide an effective means of killing oral pathogens, including *P. gingivalis* (Teanpaisan, Baxter and Douglas 1998; Kaewsrichan et al. 2004).

We have recently shown that the bacteriocin produced by *L. paracasei* HL32 has a narrow spectrum of activity against *P. gingivalis* ATCC 33277 and can cause cell death within 2 h, probably as a consequence of pore formation (Pangsomboon et al. 2006). The principal aim of the present study was to determine whether the bacteriocin isolated from *L. paracasei* HL32 exhibited antimicrobial activity against *P. gingivalis* WP 50 and other selected oral microorganisms. Antibacterial agents obtained from *L. paracasei* HL32, isolated (but not fully characterized) from a healthy volunteer, have previously been shown to have antimicrobial activity towards spoilage pathogens (Kaewnopparat 1999). *L. paracasei* has been found at the gingival crevice in healthy volunteers and has also been isolated after periodontal therapy (Koll-Klais et al. 2005). A crude product isolated from the supernatant, obtained from bacterial cultures of *L. paracasei* HL32, has been found to inhibit the growth of a range of selected microorganisms, but a narrower spectrum of the activity was demonstrated after the elimination of organic acids (Kaewsrichan et al. 2004;

Pangsomboon et al. 2006). The aim of this study was to seek to obtain a pure bacteriocin isolate and determine the structural sequence of the extracted protein. Secondary aims were to determine: (a) the mode of action of the isolated bacteriocin (b) bacteriocin cytotoxicity, as well as the stability of the isolated bacteriocin upon storage, pH and temperature changes and (c) bacteriocin activity when tested in oral biological fluids. (d) chemical synthesis of fragmented peptide from bacteriocin (e) Bioactivity studies.

MATERIALS AND METHODS

Bacteriocin production

Bacterial fermentations were carried out at 37 ^{o}C in a 5 L fermentor over a period of 72 h (MBR BioReactor AG, Switzerland). The fermentor, containing 3.6 L heat-sterilized brain heart infusion broth (BHI), was inoculated with 400 ml of an exponentially growing culture of *L. paracasei* HL32 (10^4 CFU/ml). Agitation was performed at a speed of 100 rpm to maintain the fermentation broth in a homogeneous state. Aeration was not performed, and the dissolved oxygen in the broth was depleted during the fermentation period from its initial level (4 ppm) to that present at the end (0.5 ppm) of the exponential growth phase.

Isolation, purification and identification of bacteriocin

The scheme employed to isolate the bacteriocin from the original broth culture is summarized in Fig. 1. Purification was performed using column chromatography with a Superdex-G 200 (Pharmacia, Piscatawary, NJ, USA) packed column (2 x 45 cm), using 50 mM phosphate buffer (pH 7.3) as the eluant. The isolated freeze-dried crude sample was dissolved in the eluting buffer at an initial concentration of 200 mg/ml and applied to the gel permeation column, using an ÄKTA Prime system (Amersham Pharmacia Biotech, UK) at room temperature. An elution flow rate of 1 ml/min was employed and fractions (4 ml) were collected sequentially, and monitored by UV absorption at 280 nm (Copeland 1994).

The bacteriocin-containing samples (fractions 15-25) were pooled and the resultant solutions dialyzed against distilled water (molecular weight cut-off 6-8 kDa) overnight, followed by lyophilization to generate a “partially purified” sample. A portion of this sample was dissolved (100 mg/ml) in 20 mM Tris-HCl buffer (pH 8), and this solution applied to an anion exchange column (2 x 5 cm) (HiTrap Q HP, Amersham, Pharmacia Biotech) and the sample eluted, using initially 20

mM Tris-HCl buffer at a flow rate of 1.5 ml/min. Elution was carried out using a linear gradient (from 100% 20 mM Tris-HCl buffer pH 8 at 0 min to 100% 0.5 M NaCl at 180 min) using the ÄKTA Prime system. The fractions containing inhibitory activity were observed to elute within the void volume. Some contaminants were absorbed to the anion exchange column, and these were eluted in fractions 10-78. The void volume from the column was collected and desalted using Sephadex G-25 (2 x 5 cm) (HiTrap Q HP, Amersham, Pharmacia Biotech), and the final active solid bacteriocin obtained by lyophilization. Protein content (in mg per ml) was estimated by the Lowry method (Lowry et al. 1951) using human albumin as a standard.

Gel electrophoresis

The purity of each of the isolated samples, was determined using gel electrophoresis (SDS-PAGE) (Mini-PROTEIN 3 cell, Bio-Rad) on 4-15% gradient Tris-HCl gels which employed a specified voltage (70 V) and current (222 mA) for 30 min and 1x running buffer (10x Tris/Glycine/SDS buffer, Bio-Rad). After electrophoresis, the gels were stained with GelCode® protein stain solution (Pierce, USA) for 1 h with gentle agitation. Prestained SDS-PAGE standards, which spanned a broad range of MW (7.4-192 kDa, Bio-Rad), were used as molecular weight markers.

MALDI-TOF MS/MS

The purified bacteriocin, which ran on the SDS–PAGE as a single band corresponding to a MW of 56 kDa, was excised from the gel. The process employed involved destaining the gel band, reduction with dithiotheritol and alkylation of the thiols with iodoacetamide following by in gel tryptic digestion and extraction of the resulting peptides (Millipore Singapore Ltd, Singapore). Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS) was carried out using cyano-4-hydroxycinnamic acid as a matrix. Trypsin autodigestion masses were utilised as an internal standard and the masses obtained were compared to in silico digestion of all the proteins in the database of deposited sequences using the Mascot search program (http://www.matrixscience.com), and the NCBI BLAST program (http://www/expasy.org/tools /blast) and probability-based scoring systems were used to determine the closest protein matches.

N-terminal sequencing of Bacteriocin

The sequence of amino acids in the bacteriocin was determined from the N-terminus by Edman degradation with an Applied Biosystems model Precise 492 HT automatic sequencer (Applied Biosystem). Phenylthiohydantoin-amino acids were detected at 269 nm after separation on a reverse-phase C18 column (0.46 × 25 cm) under isocratic conditions, according to the manufacturer's instructions. The sequence obtained was then compared with the protein database available in the nonredundant NCBI database using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/ Blast.cgi).

Antimicrobial activity and mode of action

The bacteria used for the antimicrobial activity testing were grown under the conditions specified in Table 1. The cylinder plate method and microtiter assay were applied to detect the inhibitory and bactericidal activities, as described previously (Javadpour et al. 1996; Koo et al. 2000).

The effect of bacteriocin on the ultrastructural morphology of *P. gingivalis* was assessed. *P. gingivalis* ATCC 33277 and *P. gingivalis* WP 50 grown in supplemented BHI broth (sBHI) were treated with the minimum bactericidal concentration (MBC, 0.14 mM in purified water) of bacteriocin for 2 h. Test bacteria were isolated by centrifuging the cultured cells at x13,400g for 5 min. Sedimented cells (pellets) were used for transmission electron microscope (TEM) preparation by fixing with 2.5% (v/v) glutaraldehyde, post fixing with 1% (v/v) osmium tetroxide for 1 h and dehydration with ethyl alcohol. The cells were then embedded in epoxy resin and a series of sections cut. These were mounted on 300 mesh copper grid, stained with uranyl acetate and lead citrate, and then examined using analytical TEM (JEOL JEM-2010 at 200 kV, Japan).

Potassium leakage

P. gingivalis (10^4 CFU/ml) was grown in sBHI (5 ml) in the absence (control) or presence of bacteriocin (at the MBC (0.14 mM)) in a series of tubes. At periodic time intervals (3, 4, and 6 h), a matched pair of cultures was removed for each time interval from incubator and the tube and contents centrifuged at x4,000g for 15 min. The potassium concentration within the supernatant was determined by atomic absorption spectroscopy (Perkin-Elmer HGA 800, Norwalk, CT), using

the method described previously (Srichana et al. 2005). Standard potassium chloride solutions (10, 20, 30, 40, 50, and 100 mg/l potassium) were prepared to check the linearity of the analytical method. Freshly prepared standard solutions were used for every experiment. Data were generated from three separate experiments.

Subjects for biological activity study

The study groups comprised 12 chronic periodontitis patients (age 53 $\pm$ 8.7, mean $\pm$ SD) and 18 periodontally healthy subjects (age 43 $\pm$ 9.8, mean $\pm$ SD). Both groups had no systemic diseases or xerostomia, and were not receiving antibiotic medication, nor had periodontal therapy within 6 months prior to the start of this study. The diagnosis of chronic periodontitis patients required the appearance of gingival inflammation, periodontal breakdown with a pocket depth 5 mm, and radiographic evidence of bone loss. Healthy individuals were designated if there was no evidence of a periodontal pocket or attachment loss, and a gingival index score was 0 or 1. Informed consent was obtained from all subjects, in accordance with the study design approved by the Ethics Review Committee on Human Research of the Faculty of Dentistry, Prince of Songkla University.

Paraffin-stimulated whole saliva was collected from the volunteers, using the method described previously (Johansson et al. 1994) and was stored at -20^{o}C until further investigation. Crevicular fluid samples were collected from different pocket sites in each chronic periodontitis patient and from two sites in each healthy subject by paper strip insertion. Prior to sampling, the area was isolated with cotton rolls, and the supragingival region of the tooth surface was cleaned and dried with sterile cotton pellets. After leaving the paper strips at each site for 1 min, each strip was transferred to an Eppendorf vial containing 1 ml of autoclaved distilled water. The vials were stored at -20^{o}C until further investigation.

Stability of bacteriocin in biological fluids

Saliva and crevicular fluid were sterilized by filtration through a 0.22 μm cellulose acetate membrane filter (Sartorius, Germany). Purified bacteriocin was dissolved in sterilized water and used as a reference, or in studies with either fluid at the level of the MBC for the study groups. The bacteriocin (0.28 mM, 100 μL) was incubated with biological fluids (100 μL) for 24 h. Percentage inhibition of *P. gingivalis* ATCC 33277 of bacteriocin in biological fluids was determined

using the microtiter assay for the antimicrobial activity, as described previously (Pangsombooon et al. 2006).

Red blood cell toxicity

Red blood cell toxicity was determined by assessing the hemolytic activity using a published method with some modifications (Kondejewski et al. 1996). In brief, bacteriocin 100 µl (0.14, 0.28 and 0.56 mM) in 10 mM phosphate buffer saline (PBS) pH 7.4 were incubated with 50 µl of 1/25 packed volume of human red blood cells distributed in microtiter plate wells. The plates were incubated with rocking (Rocker platform, Bellco Biotechnology, USA) at 37°C. Concentrations that induced either partial or complete lysis were determined visually after incubation for up to 24 h. The percentage hemolysis was calculated using equation 1. Solutions of melittin in PBS (concentrations: 0.125, 0.25, 0.5, 1.0, 2.0, 2.5 and 5 µM) were used as a positive control.

$$\%\text{hemolysis} = \left(\frac{\text{A exper} - \text{Acontrol}}{\text{Atotal} - \text{Acontrol}}\right)\text{x}100\ \% \qquad \text{Equation 1}$$

where Aexper is the absorbance values of supernatants from treated red blood cells

Acontrol is the absorbance values of supernatant from non-treated red blood cells

Atotal is the absorbance of the red cells treated with 0.1% Triton X-100, corresponding to 100% lysis

Mammalian cell toxicity

The toxicity of peptides was also assessed by determining any induced cell death of either human fibroblasts or human periodontal ligament cells (PDL). A stock peptide solution (50 µl) was diluted with an equal volume of Minimum Essential Medium (MEM), and 1:2 serial dilutions in MEM were prepared. Each dilution was applied to a 1-day old monolayer of the cells (approximately 1 x $l0^4$ cells/well) maintained in a 96-well plate with fresh MEM (50 µl/well). Peptide-treated and control (no peptide) cells were incubated at 37°C for 24 and 48 h. The supernatant was removed and the cells were gently treated with 0.2% trypan blue stain and viewed under an inverted light microscope. Inclusion of trypan blue dye within a cell is an indication of cell death. If cell population survival remained unchanged from controls, it was concluded that the peptides were not cytotoxic (Javadpour et al. 1996).

Thermal stability of the bacteriocin

The purified bacteriocin was redissolved in sterilized water and was then incubated at fixed temperatures of 25º (control), 50º, 80º, and 100 °C. Samples were removed after 5, 10, 15, 20, 25 and 30 min. The purified bacteriocin was also autoclaved at either 100 °C for 60 min, 110 °C for 30 min, or 121 °C for 15 min, 12 and 10 min. After heat treatment, the samples were cooled to room temperature and tested for antibacterial activity using the microtiter assay (Javadpour et al. 1996).

pH stability of the bacteriocin

To determine the effect of pH on the bacteriocin activity, the purified product was redissolved (at 0.14 mM) in either 5 mM phosphate-citrate buffer (pH 6.8 and 7.4) or 5 mM Tris-HCl (pH 8.5) and incubated at room temperature for 1 h. Bacteriocin was dissolved in purified water (pH 6.8 $\pm$ 0.2) for use as a reference. Antibacterial activity was subsequently determined using the cylinder plate method (Koo et al. 2000). Data were generated from three separate experiments.

Stability of the bacteriocin to enzymes

A 500 µl aliquot of bacteriocin in water (0.28 mM) was incubated with 500 µl α-chymotrypsin (500 µg/ml in 0.01 M phosphate buffer, pH 7.8), trypsin (500 µg/ml in 0.01 M phosphate buffer, pH 7.6), lipase (1 mg/ml in 0.01 M phosphate buffer, pH 7.2) or amyloglucosidase (30 mg/ml in 0.05 M citrate buffer, pH 4.5) at room temperature (25 °C) for 2 h for all enzymes, except lipase, which was incubated at 37 °C for 2 h. Following incubation, the mixture was heated at 100 °C for 10 min to denature the enzymes, and the mixture was then filter sterilized. Antibacterial activity was then determined using the cylinder plate method as described previously (Koo et al. 2000).

Storage stability of the bacteriocin

Purified bacteriocin dried solid samples (32 mg) were stored in sealed amber bottles and incubated under desiccation at 4 °C, room temperature (25 °C) or at 37 °C. A series of samples were prepared at time zero and stored at the different temperatures and individual bottled samples

were withdrawn periodically for analysis up to 6 months. Antibacterial activity in these samples was determined using the microtiter assay (Javadpour et al. 1996).

Chemical synthesis, purification and characterization

Peptide was synthesized using classical F_{moc} 9-*N*-[9-fluorenyl]-methoxycarbonyl) solid phase synthesis using a commercial automatic peptide synthesizer by coupling F-moc-alpha-amino acids on preloaded Wang resin. Protected amino acids were coupled by in situ activation with diisopropylethylamine (DIEA) and *N*-hydroxybenzotriazole (HOBt). N-Fmoc deprotection was performed with 20% piperidine in dimethylformamide (DMF). Side chain deprotection and cleavage of the peptide from the solid support were performed treatment with reagent (95%trifluoroacetic acid, 2.5% triisopropylsilane and 2.5% water) for 2 h at 20C. The peptide was purified by reversed phase HPLC using a Waters semi-preparative HPLC system. The mobile phase containing 0.065% trifluoroacetic acid in water and 0.05% trifluroacetic acid in actetonitrile as a gradient elution over 50 min (5-95%). The detector was set at a wavelength of 214 nm. The detailed gradient elution of the mixture appeared on the chromatogram with a flow rate of 1 ml/min. The peptide was eluted as a major peak. The mass spectrum analysis was carried out using electrospray ionization. Briefly, Analyses were performed with the HPLC surveyor chain connected on line to an orthogonal electrospray source operated in a positive electrospray ionization mode (ESI+). The ions were focused into an ion trap, capable of MS analyses. The mass spectra were acquired during 35 ms during 100-2000 m/z. The capillary exit of the electrospray ion source was set at 1.5 KV and capillary temperature at 250C. A counter flow of nitrogen was used as a nebulizing gas. Xcaliber data was used to acquire the data. The synthetic peptide was resuspended in 0.1% acetic acid in water and injected onto a C18 column with a 50%methanol in water at a flow rate of 0.2 ml/min. The MS data was acquired in a scan mode considering a positive ion signal. The collected fraction of purified peptide was lyophilized and used for further characterization. They were kept at 5 C for long term stability and bioactivity studies.

Bioactivity studies

When the pore forming action of the bacteriocin from previous was confirmed with periodontitic bacteria (*P. gingivalis*). Nine fragments of synthetic peptides was tested for their antibacterial activities with all oral microbiota in the previous section. If the peptide is active against

P. gingivalis, further analysis for morphogenic damage inside the bacterial cells will be carried out and observed under TEM.

Structure optimization

After peptide was chemically synthesized and chromatographically purified. The active peptide sequence was modeled based on energy minimization analysis. Energy minimization was performed on Hyperchem (Gainesville, FL,USA) for peptide-7 (Pep-7). The energy was decreased dramatically after minimization. The total energy was found as a result of decreased potential of van der waal and electrostatic interaction. The number of iterations was set for 100 for each run and continued until the energy is stable.

Statistical methods

Bacteriocin activity before and after treatments was analyzed using the Mann-Whitney U test. The differences in the antibacterial activity, after dissolving the bacteriocin in the biological fluids collected from either the healthy or periodontitis groups, were tested using the Wilcoxon signed Rank test for paired comparisons. *P-values* < 0.05 were considered statistically significant and Prism statistic software (Release 3.0) was used for this analysis.

RESULTS

Purification and identification

SDS-PAGE electrophoresis of the crude sample showed two major bands with a MW between the range of 112-116 kDa, and minor protein bands corresponding to smaller molecular weights (Fig. 2). Further purification involving elution of the crude sample through a Superdex column resulted in isolation of a 'part-pure' sample, with SDS-PAGE showing that the two major bands were still present. However the other species which produced minor bands corresponding to a MW of 16-45 kDa had been eliminated. The final 'purified' product, obtained after passing through an anion exchange column, exhibited a single band by electrophoresis, corresponding to 56 kDa and the protein retained antimicrobial activity.

After tryptic digestion, MALDI-TOF MS of the 'pure' bacteriocin in the positive mode gave species with masses of 800.31, 807.32, 808.31, 809.31, 812.31, 820.31, 822.31, 835.33, 847.34, 859.33, 1002.66, 1028.65, 1046.66, 1046.66, 1048.44, 1171.60, 1174.62,

1190.62, 1192.63, 1208.63, 1224.62, 1236.69, 1255.85, 1283.72, 1365.73, 1396.83, 1422.81, 1438.81, 1439.82, 1454.81, 1456.82, 1456.82, 1472.81, 1472.81, 1599.87, 1716.95, 1760.97, 1765.86, 1777.97, 1782.05, 1782.05, 1800.06, 1800.06, 1801.05, 1837.01, 1882.05, 1987.20, 2009.13, 2131.13, 2145.27, 2157.19, 2158.18, 2159.17, 2163.28, 2163.28, 2176.15, 2190.13, 2221.25, 2230.33, 2233.25, 2263.29, 2297.33, 2408.20, 2564.33, 2618.47, 2618.47, 2691.45, 2709.47, 2723.49, 2825.49, 2941.67, and 3346.90 m/z, respectively. In Table 2, the underlined species were singly charged ions and the net profile was consistent with the bacteriocin primary sequence obtained by the Edman degradation as following:

1 AEPGLFGTITGAMYREGQHKRLVAKPVFAQRVPAIPSGLQRPQGRDGPGQ 50

51 RPHGAGEGIDRVPAGPSPSEVGLAIPSGKQAGPVGRQNATGWKGPSKSQP 100

101 KSGPSPEPKPQNHGPHGDAGNTEANGGEGPSNTGEPPGSARNNPDNAPAG 150

151 AGGGGA

No residue could be determined at position 157-160 presumably due to wash out of the sample from the sequencer. The amino acid composition from primary sequence was also consistent with the amino acid content obtained by analysis. When the primary sequence corresponding to bacteriocin were searched against the SWISSPORT and Protein Data Bank databases using both FASTA and BLAST. The bacteriocins revealed a low score of matching, the values, from highest to lowest, being 41.4, 40.4, 35.4, 25.4, 24.6, 24.6 bits. This sequence did not show clear homologies with known bacteriocins.

Antimicrobial activity and mode of action

The activity spectrum of purified bacteriocin against a few oral bacterial species is shown in Table 3. Metronidazole (0.1 μg/ml) demonstrated activity against all species of obligate anaerobe, whereas only the two strains of *P. gingivalis* were susceptible to the antimicrobial action of bacteriocin. The other oral pathogens and normal flora were insensitive to the bacteriocin activity.

Transmission electron micrographs of *P. gingivalis* exposed to the bacteriocin showed areas where the outer and inner membranes appeared to be disrupted at their pole, as indicated by the dark arrows in Figs. 3c and 3d, without apparent loss of the underlying cell morphology. Cell membranes of the control group (Figs. 3a and 3b) remained intact. The presence of

membrane associated vesicles, with their distinctive trilaminar appearance, was apparent in the control images, as shown by the white arrows in Figs. 3a and 3b, but the vesicles were absent in the cells treated with bacteriocin. The cytoplasm in all groups is homogenous in appearance, with no evidence of clumping or filamenting of the cytoplasmic constituents, and no phage particles were observed. There appears some cell debris in Fig. 3d as a consequence of cell lysis.

The analytical method employed to determine potassium concentration by atomic absorption produced a linear response (10-100 mg/L, r^2 = 0.990). There was an increase in potassium concentration within the supernatant when cultures of *P. gingivalis* were incubated with bacteriocin. Potassium levels increased from 32 mg/ml at 0 time to 42, 40 and 38 mg/ml at 3, 4 and 6 h, respectively with both strains of *P. gingivalis* in the presence of bacteriocin. The potassium concentration in the supernatant appeared to decrease with time and this may be due to the adsorption of ion to dead cells after 4 and 6 h. Potassium levels were essentially unchanged when bacteriocin was omitted.

Hemolysis assay

Bacteriocin was found to be non-hemolytic at the MBC under the conditions employed in this study following 24 h incubation (Table 4). When erythrocytes were treated at double the MBC, bacteriocin showed 16% relative hemolysis, compared with the positive controls of 0.1% (v/v) Triton X-100 or melittin (0.625 μM), where 100% hemolysis occurred following the incubation period.

Mammalian cell assay

No effects on the morphology and viability of fibroblast cells and periodontal ligament cells were observed for bacteriocin treatment up to the MBC (0.14 mM) after 24 h incubation (Fig. 4). A significant decrease in viable cells was observed when the concentration was increased to 0.28 mM or higher, although at shorter period of time (2 and 6 h) bacteriocin did not show any toxicity to PDL cells. The same concentrations of bactericin were better tolerated by PDL cells than fibroblasts (Fig. 4). A significant decrease in the % viable cells at higher concentrations was observed when either bacteriocin or mellitin were incubated with either cell-line. However, the

toxicity of mellitin was obviously much greater than that for bacteriocin on a weight/volume basis, given the differences in concentration of the two species.

Effects of biological fluids on bacteriocin activity

The antimicrobial activity of bacteriocin on *P. gingivalis* ATCC 33277 was not different (P > 0.05) when it was incorporated either with saliva derived from healthy volunteers or when included with saliva derived from periodontal patients (Table 4). There was also no decrease in % inhibition in either saliva or crevicular fluid when compared to inhibition induced by the control (75 ± 5 %). The % inhibition determined in the presence of gingival crevicular fluids obtained from healthy volunteers apparently decreased by 2%from the control and there was no change in case of periodontitis patients. However, there was no statistical significant difference in the reductions (*P > 0.05*) and there was no difference between the inhibition in the presence of saliva and gingival crevicular fluid obtained from the healthy volunteers (*P = 0.201*) (Table 4). It can be concluded that the presence of saliva and gingival crevicular fluids did not have any major impact on the activity of the bacteriocin.

pH, thermal, and storage stability and sensitivity to the enzymes

The bacteriocin remained soluble and active over a pH range of 6.8-8.5. A reduction in its bactericidal activity was observed following heat treatment, but even autoclaving the bacteriocin in solution at 121 ^{o}C for 15 min decreased the activity by only 4-8%. There was no statistically significant difference between the % inhibition obtained after heat sterilization at 100 ^{o}C for 60 min and that obtained following filter sterilization (*P=0.841*). There was also no significant reduction in activity upon storage of bacteriocin at 4, 25 and 37 ^{o}C for 6 months (data not shown). The antibacterial activity of the bacteriocin was destroyed by incubation with trypsin and chymotrypsin. There was no reduction in activity by incubation with lipase but there was a slight reduction in activity as a consequence of incubation with amyloglucosidase (Table 3).

Chemical synthesis, purification and characterization

All peptides were obtained to be white powder. The yield was found to be at least 70% and the chromatographic purity was confirmed (Figure 5). When peptides were injected into electro-spray mass spectrometry, major peaks corresponding to molecular ion of peptide were detected

with positive ion mode. Their mass to charge ratio (m/z) indicated a singly positive charge on molecular ion of peptide. Figure 6 demonstrates that fragment mass spectrum of the purified peptide showing peaks for $[M+4H]^{4+}$, $[M+3H]^{3+}$ molecular ions. The MW of 2809.8 corresponded to synthetic Pep-7 of RPHGAGEGIDRVPAGPSPSEVGLAIPSGK sequence. The calculated mass of amino acid sequences was 2809. It is suggested that the ionization of this peptide gives a product containing designed amino acids sequence. Molecular mass analysis of all peptides we synthesized gave the same pattern of mass spectrum as shown in the appendix and their masses are listed in Table 5.

Bioactivity

This work demonstrated that the synthetic peptides (9 peptides) are not all active against microbes, only two peptides were active against *P. gingivalis* and this was also a selective activity on pathogenic bacteria (Table 6). Peptide synthesis and bioactivity from synthesized peptides gave common characteristics in antibacterial activities. Those observations are listed as follows; Pep-7 has four cationic amino acids (2 Arg, 1 His and 1 Lys), three anionic amino acids (2 Glu and 1 Asp). The hydrophobic and hydrophilic balance are very ideal (13 hydrophobic amino acids and 9 hydrophilic amino acids). The molecule has more hydrophobic therefore, it assists in favor interaction between the molecule with the bacterial cells. Also, the cationic part may contribute to an interaction with the cell membrane of bacteria.

Structure optimization

The helical conformation are made up with a fair amount of amino acids residues of high helical propensity such as alanine (3), leucine (1), glutamic acid (2). An amphipathic propensity was contributed from glycine and serine and cationic charges are present for bioactivity (Arg, Lys and His). Anionic residues were found such as glutamic acid and aspartic acid. Aromatic residues spread along the peptide (5 residues) and perhaps may assist in molecular interaction with bacterial membrane. Peptides from 9 fragments and number of amino acids residues are varied from 9 to 35. The structure of relative hydrophobic and hydrophilic ratios was varied from 20% to 60%. Polar peptides are contributed from cationic and anionic residues. For the Pep-7, we have used energy minimization as a mean to determine whether the molecule can adapt stable conformation with standard bond length and angle (Figures 7-8). Pepe-7 seems to be stable in left

hand alpha turn or beta turn 310 alpha helix. This is possible caused by the distribution of charge and polar side chain on peptide that provide stabilization to the peptide.

DISCUSSION

Partial characterization of the bacteriocin for *L. paracasei HL32* has shown that it was composed of 26.3% hydrophobic amino acids in the total residues of 171 amino acids (Pangsomboon et al. 2006). Lactic acid, a major by-product of lactobacilli fermentation, was removed by dialysis (Schillinger and Lucke 1989; Daeschel, McKenney and McDonald 1990) and the purification procedures employed in this study have led to the isolation of a purified bacteriocin. The molecular weight of this pure bacteriocin was determined by SDS-PAGE. The crude product migrated as a protein with a MW of 112-116 kDa; however, further purification generated a single band corresponding to a MW of around 56 kDa. The latter MW was the same as that reported in a previous study, which employed electrospray ionisation mass spectrometry (ESI-MS) (Pangsomboon et al. 2006). About a half reduction in MW may be due to the fact that the dimer peptide might be broken down to monomer. The treatment of the peptide during the purification step or during ESI-MS, results in the dimer apparently being cleaved and this has been reported to occur with other bacteriocins (Mortvedt et al. 1991; Onda et al. 2003).

One hundred and fifty six amino acid residues of bacteriocin were detected by N-terminal amino acid sequencing. The sequence was not blocked by modified amino acids residues contained in the structure, such as lanthionine or acetylated derivatives, which was, similar to observations in earlier studies (Mortvedt et al. 1991; Remiger et al. 1999). Protein sequence as determined by N-terminal Edman degradation analysis was confirmed by information obtained from a combination of sequence analysis of the tryptic digestion fragment with mass spectrometry (Table 2).

Comparison of the amino acid sequence of the bacteriocin with those of other bacteriocins using FASTA and BLAST revealed very low matching to the partial sequence obtained from each resource with highest score of only 41.4. The non-homology between this bacteriocin produced from *L. paracasei* HL32 and previous isolated bacteriocins confirms that the isolated bacteriocin is a novel protein.

The amino acid sequence of the *L. paracasei* HL32 bacteriocin shows the presence of cationic amino acids near the N-terminus which has a similarity to plantaricin 1.25β (Remiger et al. 1999) and plantaricin C (Gonzalez et al. 1994) and a high percentage of hydrophobic amino acids in the central region (Leu, Ile, Val, Phe, Trp and Gly). It also contains a highly polar C-terminus (Ser, Asn and Gln). Taken together, it is possible that the bacteriocin exerts its action through interaction with cell wall-associated, or membrane-associated, binding sites in the sensitive cells. Our results suggested that the net positive charge at the 30 N-terminal amino acids (5+ at pH values lower than 6) would interact with the negative charge of phosphate group and decanoic acids in the cell wall of *P. gingivalis* cells, and may be required to attain a strong electrostatic interaction. The bacteriocin may interact specifically and competitively with some target cell entity, which would then be followed by membrane insertion of the uncharged portion of the bacteriocin, allowing the bacteriocin to form membrane pores, which increase membrane permeabilization and cause cell death. Since the sequencing revealed only one thiol containing amino acid, this peptide may not have any secondary structure. In addition, the sequencing of the bacteriocin revealed many Pro, Gly and Ser residues, after subjecting the sequence into secondary structure prediction at http://swissmodel.expansy.org, the bacteriocin appears to form a random coil further indicating the absence of a secondary structure to this peptide.

The selection of the indicator strains (Gram-positive bacteria: *Streptococcus sanguinis* and *Streptococcus salivarius*) was based on their importance in the oral ecosystem as members of the dominant indigenous normal flora. Other periodontal pathogens were included in the study to evaluate the specificity of the antagonistic substance(s). Even when metronidazole, the drug of choice in periodontal treatment, is employed, *Streptococcus mutans* and microaerophilic bacteria have been reported to increase in number after drug treatment (Loesche et al. 1991). The results of this study revealed that the *L. paracasei* HL32 produced an antibacterial compound which, when purified, inhibited *P. gingivalis* strains only. Interestingly, the bacteriocin was inactive after incubation with trypsin and chymotrypsin, but after treatment with either enzyme it showed activity against *S. salivarius*. The trypsin activity cleaves amide and ester bonds of substrate specificity between arginine (Arg) and lysine (Lys) side chains (Creighton 1993), whereas the chymotrypsin cleaves the peptide bond at tryptophan (Trp), phenylalanine (Phe), tyrosine (Tyr) and leucine (Leu) (Creighton 1993), The bacteriocin contained 9 residues of Arg, 9 residues of Lys, 2 residues of

Phe, 1 residue of Tyr and 6 residues of Leu (Pangsomboon et al. 2006). Hence, cationic and hydrophobic residues (Lys, Arg and Leu) of bacteriocin may play an important role in the observed antimicrobial activity, which is in accordance with previous observations (Montville, Winkowski and Ludescher 1992; Hancock 2002; Duche 2002; Zasloff 2002). In addition, the antibacterial activity was reduced by amyloglucosidase treatment, indicating that bacteriocin is likely to contain carbohydrates. Nevertheless, deglycosylated bacteriocin still retained bactericidal activity (87% inhibition) against both strains of *P. gingivalis*. Alternatively the amyloglucosidase preparation may have some proteolytic activity.

Potassium is the major intracellular cation in bacteria. K^+ acts as a cytoplasmic-signalling molecule, activating or inducing enzymes and transport systems that allow the cell to adapt to the elevated osmolarity (Epstein 2003). The perforation of the cell membrane and the absence of vesicles as viewed by TEM, together with the fact that cells of *P. gingivalis* incubated with the bacteriocin showed efflux of potassium, suggests that the bacteriocin targets the bacterial membrane and lipopolysaccharide. Therefore, potassium leakage might be expected to occur as a secondary event (Montville, Winkowski and Ludescher 1992). The protrusions visible in the bacterial cells indicate weaknesses in the cell walls. TEM confirmed that the bacteriocin shows bactericidal activity against *P. gingivalis*. The absence of vesicles is an advantage because such vesicles contain many virulence factors related to the onset and progression of periodontal disease (Smalley and Birss 1991; Srisatjaluk, Doyle and Justus 1999; Duncan et al. 2004). It is interesting to further investigate the factor involving in an absence of vesicles.

The erythrocyte plasma membrane is a natural membrane in the body which contains anionic surface charges and a lipid monolayer. In this study the toxicity of bacteriocin was compared with that of melittin, a venom peptide derived from the bee. The bacteriocin was shown to induce hemolysis only at very high concentrations (2-4 fold higher than the MBC). In the cell cytotoxicity assay for the bacteriocin, the periodontal cells and fibroblast cells survived after 24 h even in presence of MBC of bacteriocin. At high bacteriocin concentrations (0.28, 0.56 mM), cell survival was time dependent. However, different toxicities of the bacteriocin to the periodontal and fibroblast cells were observed, when the concentration of the bacteriocin was above 0.14 μM. Therefore, the bacteriocin cytotoxicity appeared to be concentration- and time-dependent.

Bacteriocin activity may be reduced by the action of enzymes or may be influenced by pH, temperature, and biological fluid. A previous study has demonstrated that pH values from 2.6 to 5.6 inhibited the growth of *P. gingivalis* without any treatment (Takahashi et al. 1997; Kaewsrichan et al. 2004). However, if the concentration of buffer was reduced from 50 to 5 mM, the different pH values were found not to affect the growth of *P. gingivalis*. Over a pH range of 6.8 -8.5, using the lower buffer concentration, there was no change in the antimicrobial activity of the bacteriocin. Therefore, it may be practical to use this bacteriocin as a topical application in the subgingival pocket because bacteriocin is active over pH range of 6.8-8.5 while *P. gingivalis* strains grow at neutral pH, and the pH tends to rise to alkaline conditions during growth (Takahashi et al. 1997). In respect to temperature sensitivity, cell free supernatant samples were used in early studies (Daeschel, McKenney and McDonld 1990; Sobrino et al. 1991; Suma, Misra and Varadaraj 1998) but the present study has utilized purified bacteriocin. The bacteriocin appeared to be heat-stable, since it was able to withstand the effects of temperatures over 100^{o}C for 1 h, with no effects on residual antibacterial activity. Such heat stability is unusual, but is probably a result of protein glycosylation, which generally provides resistance against degradation by heat treatment. This heat stability would be advantageous for possible commercial development of the bacteriocin, since sterilization by autoclaving could be contemplated instead of the more expensive filtration process. The bacteriocin resistance to autoclaving indicates that the antibacterial inhibitory effects are not a consequence of bacteriophage contaminant. In this investigation, the antimicrobial activity of bacteriocin was not markedly affected by saliva or gingival fluids.

The bacterial activity of Pep-7 was confirmed from Table 6. Pep-6 has similar properties to Pep-7 but has much less antibacterial activities. This needs to be further analysis for its specific activities of Pep-7 against P. gingivalis. The TEM was done to confirm the mechanisms of bacterial cell damage it revealed similar observation as that obtained with larger peptide bacteriocin. In such a way we can deal with a smaller and shorter peptide and it\is feasible to synthesize in the laboratory. When we can scale up the production of Pep-7 we will confirm the toxicity studies with periodontal ligament, fibroblast and red blood cells. From preliminary studies we did not find toxicity to red blood cells at the concentration that was able to kill the *P. gingivalis*.

CONCLUSION

In summary, the isolated bacteriocin from *L. paracasei* HL32 was shown to have selective antimicrobial activity against two strains of *P. gingivalis*. However, its MBC is quite high which may present problems in its development for clinical use. A bacteriocin that allows selective killing of a specific pathogen, without disturbing some normal oral microflora in this study, and has no toxicity at therapeutic level would be a good candidate for periodontal treatment. However, the bacteriocin from *L. paracasei* HL32 would appear not to be suitable for systemic administration, since it does exhibit toxicity to human erythrocytes. However, it may be possible in future studies to use the bacteriocin as a starting compound and produce derivatives with better activity profile, lower MBC and lower toxicity.

The selective bioactive peptide was assayed and found to be stable and active against P. gingivalis. From peptide synthesis of this active fragment and the active peptide is new it is likely to be able to patent this sequence. This work has proved to obtain a p[roduct from biosynthesis and chemical synthesis active peptide. This two methods synergized one another tool to obtain an active peptide. Our attempt by the design based on structure activity relationship does not help to achieve active peptide. In the future we hope to understand more about the mechanism of this peptide against the microbe to be able to improve the bioactivity. Other things is the scale up production of peptide at a reasonable cost for future clinical studies.

ACKNOWLEDGEMENTS

The authors would like to thank the Commission of Higher Education and the Thailand Research Fund (grant no. RMU 4980041) for financial support and Prince of Songkla University for the use of facilities and in particular S. Kamolmattayakul and N. Wattanaarunwong (Prince of Songkla University) for the gift of the human fibroblasts and human periodontal ligament cells used in these studies. The matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was carried out at the Protein and Proteonomic Center, Department of Biological Sciences, National University of Singapore. We also wish to thank to Prof. L.A. Damani for editing our manuscript.

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Table 1 Bacterial strains and growth conditions

Organism	ATCC number	Media		Conditions	
		Broth	Agar	Temperature	Atmosphere
Porphyromonas gingivalis	33277™	sBHI (BHI with YE 5 mg/ml, vitamin K_1 5 μg/ml and hemin 5 μg/ml)	TSA with YE 1 mg/ml, vitamin K_1 5 μg/ml, hemin 5 μg/ml and 5% blood	37°C	Anaerobic gas mixture*
Porphyromonas gingivalis	53978™				
Prevotella intermedia	25611™				
Tannerella forsythensis	43037™				
Streptococcus sanguinis	10556™	BHI	TSA with 5% blood	37°C	5% CO_2
Streptococcus salivarius	25975™	BHI	BHI agar	37°C	Aerobic

Brain heart infusion broth (BHI); supplemented BHI (sBHI); tryptic soy agar (TSA); yeast extract (YE)

* Composition of anaerobic gas mixture (80%N_2 + 10%CO_2 + 10%H_2)

Table 2 Relationship between protein sequences obtained from Edman degradation and the calculated masses

Protein sequence obtained from Edman degradation	Residue X to Y	MALDI-TOF (m/z)	Mr (calc)
AEETLMTEYTA (former sequence)	1 to 11	1255.85	1257.533
AEPGLFGTITGAMYR+1(M)	1 to 15	1599.87	1598.773
AEPGLFGTITGAMYREGQHK+1(M)	1 to 20	2176.15	2178.105
REGQHKRLVAK	15 to 25	1208.63	1207.679
RLVAKPVFAQR	21 to 31	1283.72	1283.772
RVPAIPSGLQR	31 to 41	1192.63	1192.693
RPQGRDGPGQR	41 to 51	1224.62	1222.617
RVPAIPSGLQRPQGRDGPGQR	31 to 51	2263.29	2261.062
RPHGAGEGIDRVPAGPSPSEVGLAIPSGK	51 to 79	2825.49	2825.457
KQAGPVGR	79 to 86	812.31	811.4559
RQNATGWKGPSK	86 to 97	1171.6	1172.583
KSQPKSGPSPEPK	97 to 109	1365.73	1365.715
SKSQPKSGPSPEPK	96 to 109	1454.81	1452.747

Table 3 Antimicrobial activity spectrum of bacteriocin produced by *L. paracasei* HL32, compared with metronidazole as a reference

Bacterial strains	Mean (±S.D.) zone diameters of inhibition (mm), n = 4					
	metronidazole 0.1 μg/ml	Bacteriocin (0.14 mM) treated with enzyme				
		Enzyme				
		no enzyme	Trypsin	Chymo-trypsin	Lipase	Amyloglu-cosidase
P. gingivalis ATCC 33277	48 (0.5)	25.5 (0.3)	(-)	(-)	24.8(0.2)	17.2 (0.3)
P. gingivalis WP 50	16.6 (0.2)	19.8 (0.4)	(-)	(-)	19.2 (0.3)	17.3 (0.2)
P. intermedia ATCC 25611	53.6 (1.1)	8.0 (0.2)	(-)	(-)	(-)	(-)
T. forsythensis ATCC 43037	70 (0.3)	(-)	(-)	(-)	(-)	(-)
S. salivarius ATCC 25975	(-)	(-)	14.1(0.2)	17.1 (0.2)	(-)	(-)
S. sanguinis ATCC 10556	(-)	(-)	(-)	(-)	(-)	(-)

– , no inhibition zone

Table 4 Hemolytic effect of HL32 bacteriocin and the effect of heat, pH and biological fluids on the activity of the bacteriocin

Treatment (time)	n	% inhibition (±SD)	% hemolysis (±SD)	*P-value*
Bacteriocin 0.14 mM (24h)	3	83(0)	0	-
0.28 mM (24h)	3	100	16 (0)	-
0.56 mM (24h)	3	100	100	-
Melittin 0.625 mM (24h)	3		100	-
Bacteriocin 0.14 mM				
pH: 6.8 (1h)	3	83 (4)		-
7.4 (1h)	3	84 (1)		-
8.5 (1h)	3	83 (4)		-
Heat:				
100°C for 60 min	4	84 (2)		0.841
110°C for 30 min	4	80 (0)		0.007
121°C for 10 min	4	80 (2)		0.007
121°C for 12 min	4	76 (5)		0.015
121°C for 15 min	4	75 (4)		0.015
Biological fluid (time 24h)				
A, Saliva (healthy volunteers)	3	67 (7)		A and C, 0.2016
B, Saliva (periodontitis patients)	3	72 (10)		B and A, 0.1484
C, GCF (healthy volunteers)	3	73 (6)		C and D, 0.4609
D, GCF (periodontitis patients)	3	75 (6)		D and B, 0.3828
Control (0.14 mM in PBS)	3	75 (5)		

Porphyromonas gingivalis ATCC 33277 was used as an indicator organism.

Table 5 Synthesized peptides from fragmented bacteriocin

Peptide	Protein sequence obtained from chemically synthesis	MW (found)	MW (calc)
Pep-1	RLVAKPVFAQR	1284.56	1283.77
Pep-2	RPQGRDGPGQR	1223.31	1222.61
Pep-3	REGQHKRLVAK	1321.54	1207.68
Pep-4	AEPGLFGTITGAMYR AEPGLFGTITGAMYREGQHK	3728.30	3729.26
Pep-5	RVPAIPSGLQR	1193.30	1192.69
Pep-6	RVPAIPSGLQRPQGRDGPGQR	2242.50	2261.50
Pep-7	RPHGAGEGIDRVPAGPSPSEVGLAIPSGK	2809.13	2825.45
Pep-8	KQAGPVGR RQNATGWKGPSK	2123.39	2123.39
Pep-9	KSQPKSGPSPEPK SKSQPKSGPSPEPK	2802.11	2802.11

Table 6 Molecular shape and minimized energy of Pep-7

Shape	Minimized energy
Beta sheet	24.326
Anti-parallel beta	-2.200
Parallel beta	-57.166
Pi- helix	-57.059
310 helix	-113.474
Alpha helix	-140.292
Beta turn	-261.170
Left hand alpha	-139.994

Table 7 Bioactivity of synthetic peptides against pathogenic and mormal flora in oral cavity

organism	Inhibition zone(mm)										
	Pep-1	Pep-2	Pep-3	Pep-4	Pep-5	Pep-6	Pep-7	Pep-8	Pep-9	Tet	Metro
P. gingivalis 33277	-	-	-	-	-	8	20.25	-	-	38	15
P. gingivalis 53978	-	-	-	-	-	7.25	20.37	-	-	40	12
P. intrrmedia 25611	-	-	-	-	-	-	-	-	-	38	20
T. forsythensis 43037	-	-	-	-	-	-	-	-	-	40	20
S. sanguinis 10556	-	-	-	-	-	-	-	-	-	40	18
S. salivarius 25975	-	-	-	-	-	-	-	-	-	45	15
S. sureus 25923	-	-	-	-	-	-	-	-	-	38	12
E. coli 25922	-	-	-	-	-	-	-	-	-	39	15

- = No inhibition zone

Tet = Teracycline (conc)

Metro = Metronidazole

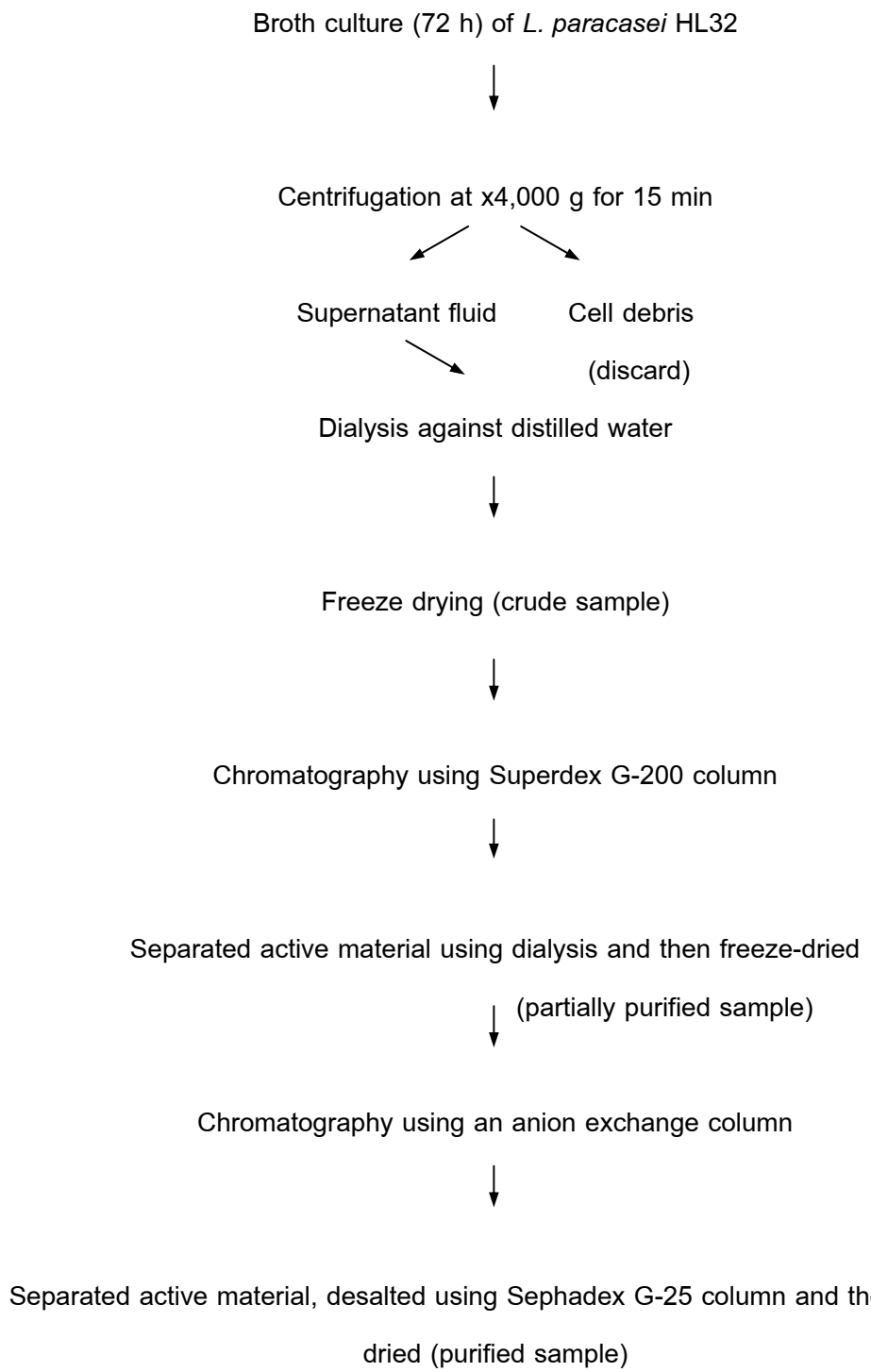


Figure 1 Isolation and purification of bacteriocin produced by *Lactobacillus paracasei* HL32 in 72 h broth culture

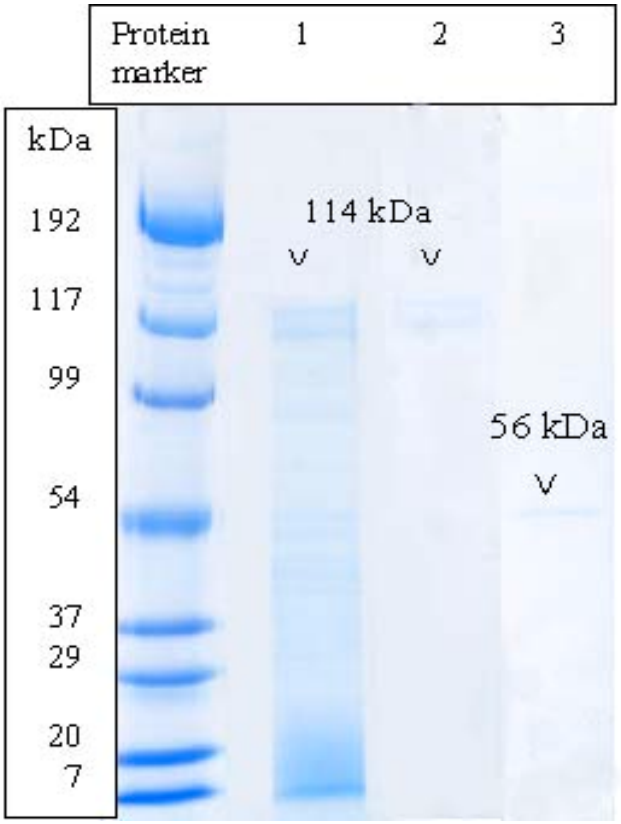


Figure 2 SDS-PAGE of bacteriocin produced by *L. paracasei* HL32 on 4-15% Tris-HCl gel. Crude sample (Lane1), partially purified sample (Lane 2) and purified sample (Lane 3).

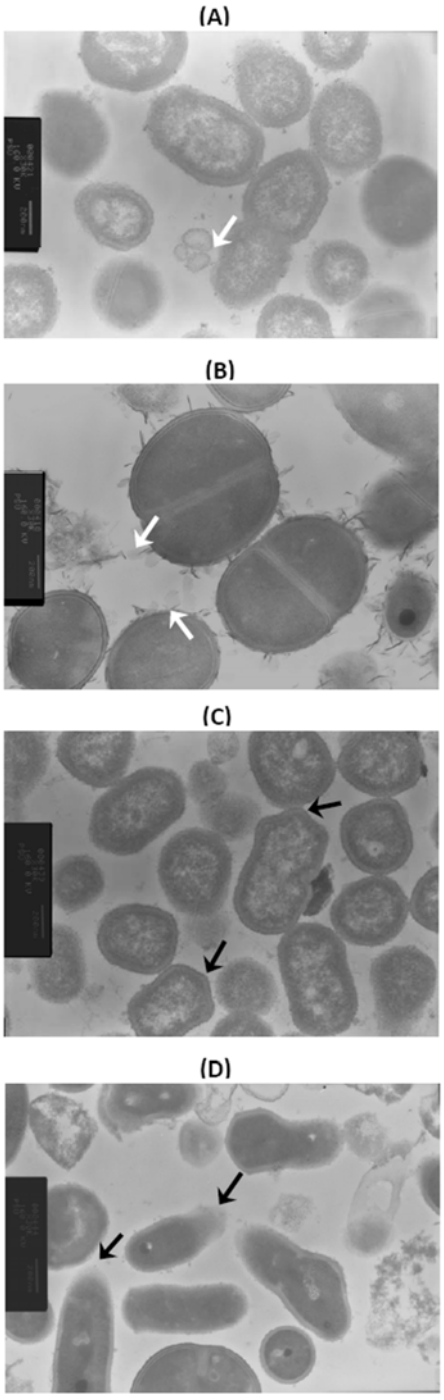


Figure 3 Transmission electron micrographs (TEM) of *P. gingivalis* (A) ATCC 33277 control (B) WP 50 control (C) ATCC 33277 incubated with 0.14 mM bacteriocin (2 h) and (D) WP 50 incubated with 0.14 mM bacteriocin (2 h). Bar = 200 nm in all figures.

White arrows indicate vesicles in 3a and 3b and dark arrows indicate changes in bacterial cells in 3c and 3d.

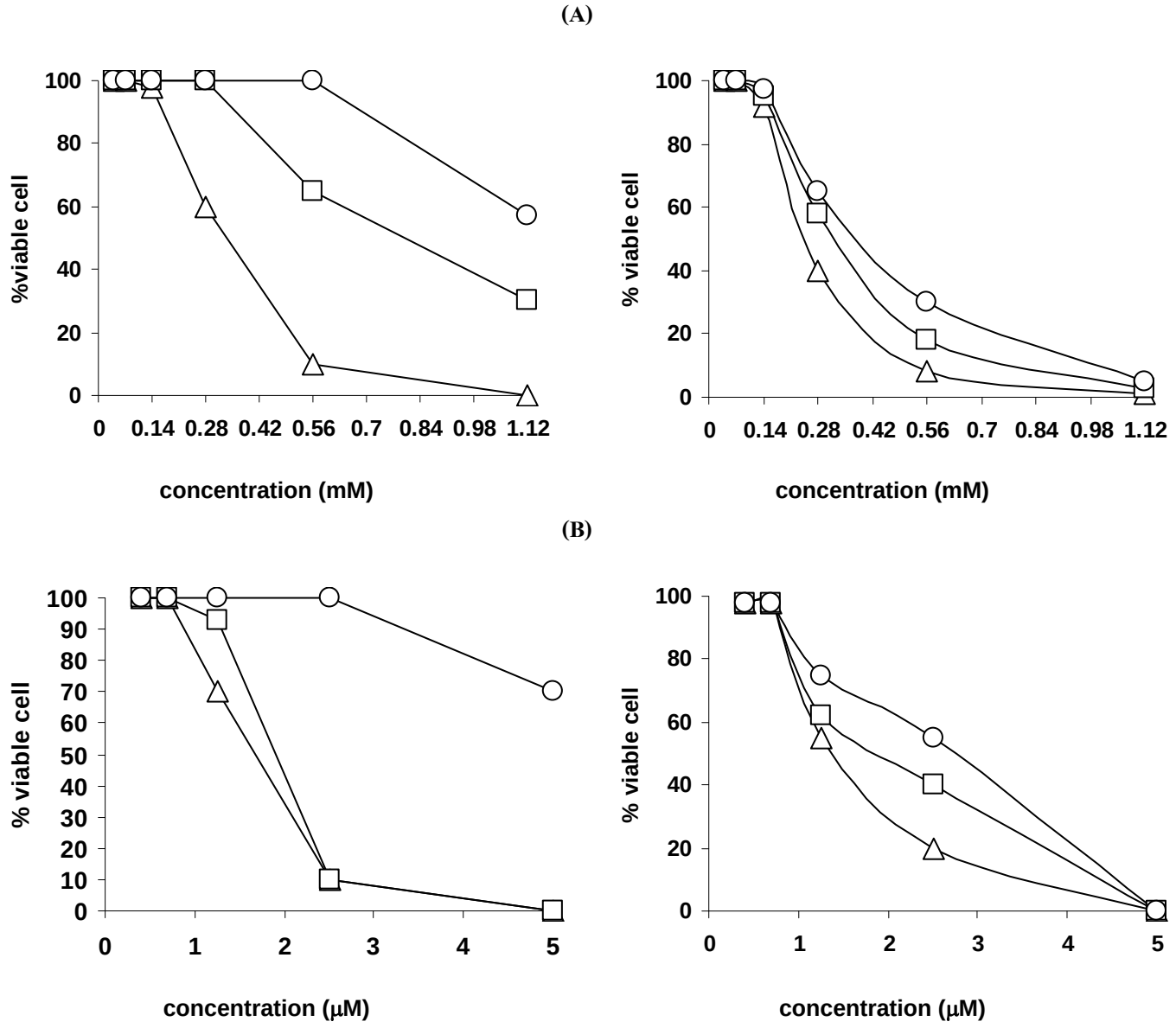


Figure 4 The effect of bacteriocin (A) and mellitin (B) on viability of human periodontal ligament cells (left) and fibroblasts cells (right) after incubation for 2 h (○), 6 h (□) and 24 h (△) (mean ±SD, N=3)

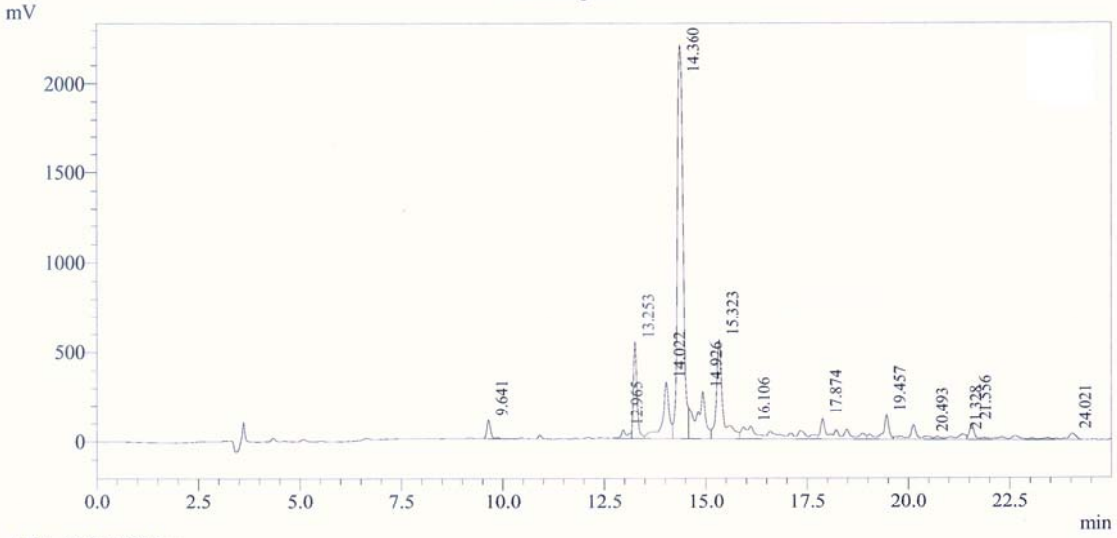
Sample Name : AE
Sample ID : 64461-4

Pump A : 0.065% trifluoroacetic in 100% water
Pump B : 0.05% trifluoroacetic in 100% acetonitrile
Total Flow : 1 ml / min
Wavelength : 214 nm

Time	Unit	Command	Value	Comment
0.01	Pumps	Pump A B.Conc	5	
25.00	Pumps	Pump A B.Conc	65	
25.10	Pumps	Pump A B.Conc	95	
35.00	Pumps	Pump A B.Conc	95	
35.10	Pumps	Pump A B.Conc	5	
47.00	Pumps	Pump A B.Conc	5	
47.10	Controller	Stop		

<Detector A>
Column : X Bridge 4.6 x 250mm

Chromatogram



1 Det.A Ch1 / 214nm

Peak Table

Detector A Ch1 214nm

Peak#	Ret. Time	Area	Height	Area %
1	9.641	672527	103374	1.297
2	12.965	533469	47637	1.029
3	13.253	3317675	545570	6.397
4	14.022	3756396	320794	7.243
5	14.360	21958102	2195909	42.340
6	14.926	4383790	267125	8.453
7	15.323	6049140	554549	11.664
8	16.106	3466593	70868	6.684
9	17.874	2328034	113616	4.489
10	19.457	1415622	134634	2.730
11	20.493	1183207	17181	2.281
12	21.328	725162	29809	1.398
13	21.556	1435343	85645	2.768
14	24.021	636783	35684	1.228
Total		51861843	4522394	100.000

Figure 5 Chromatogram of Pep-7 from peptide purification

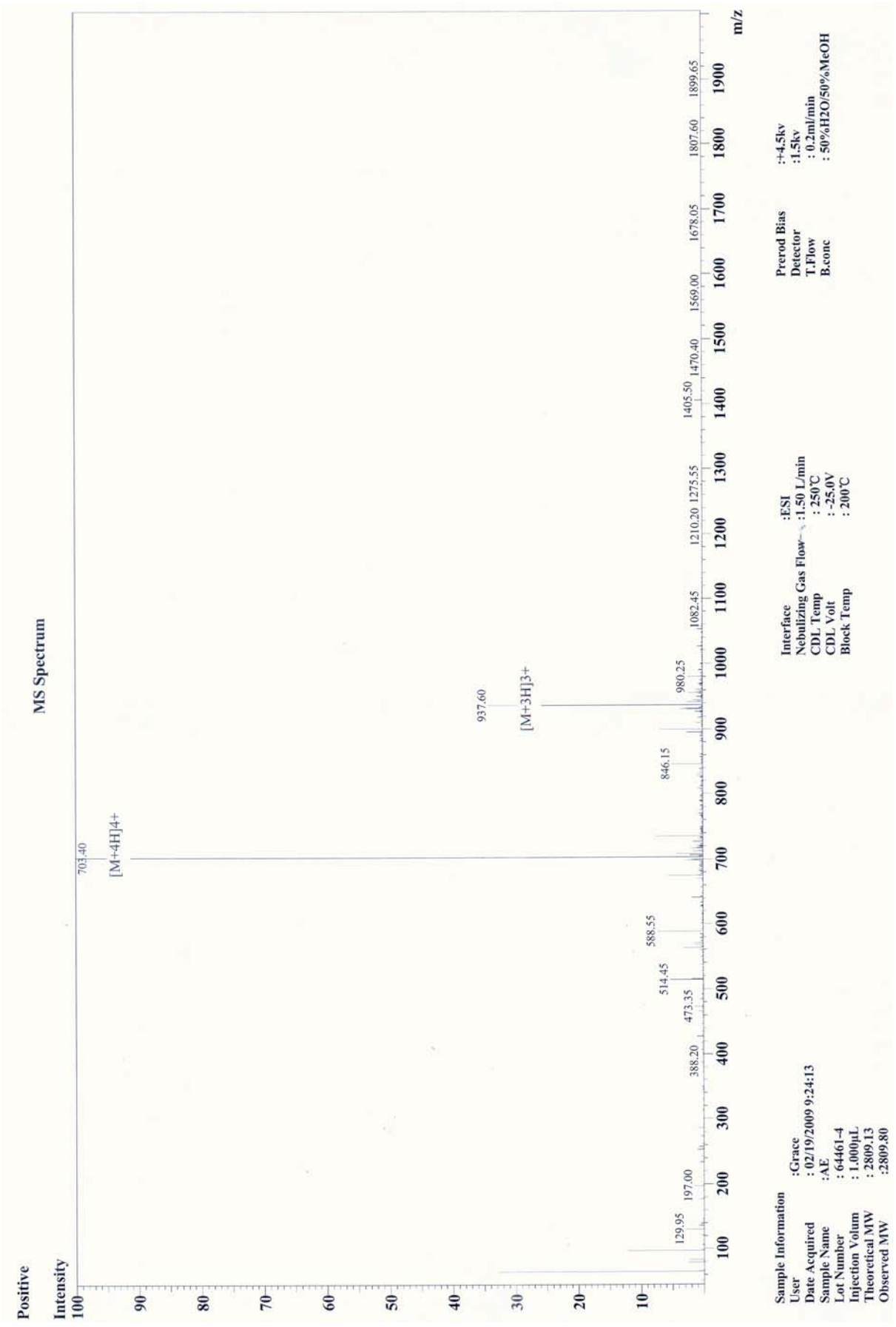


Figure 6 Mass spectrum of Pep-7

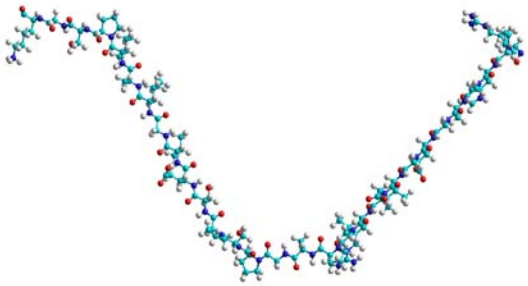
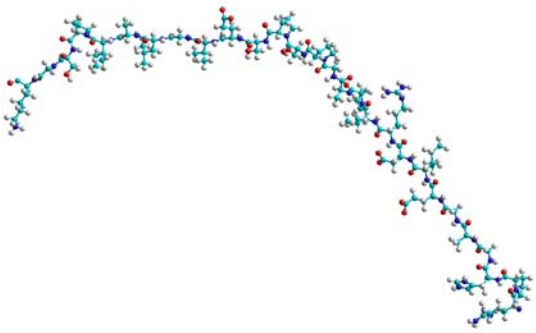
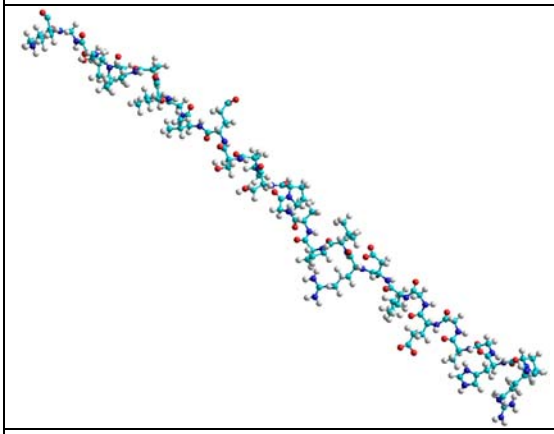
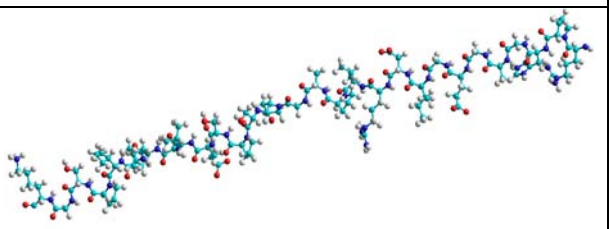
	
Beta sheet	Antiparallel beta
Energy optimization = 24.326	Energy optimization = -2.200
	
Paraell beta	Pi helix
Energy optimization = -57.166	Energy optimization = -57.059

Figure 7 Structural representation of Pep-7 (RPHGAGEGIDRVPAGPSPSEVGLAIPSGK) contributed from constructed from standard parameters of beta sheet, antiparallel beta, parallel beta and pi helix.

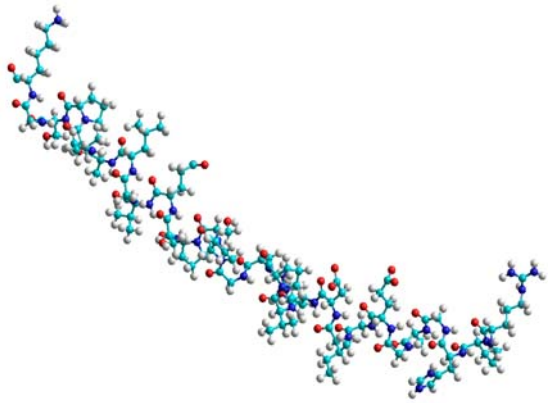
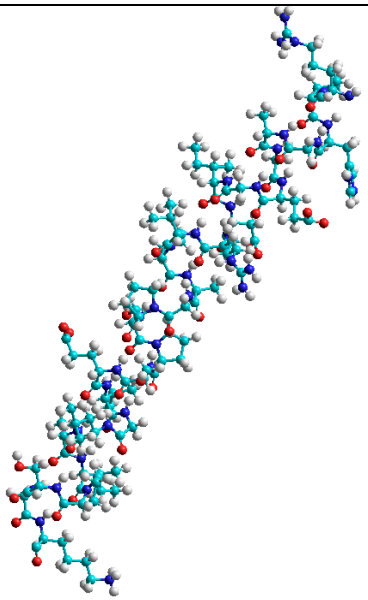
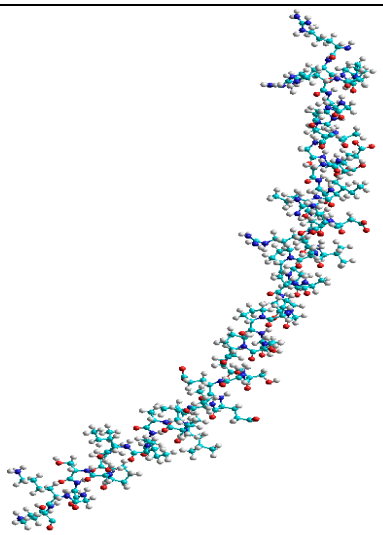
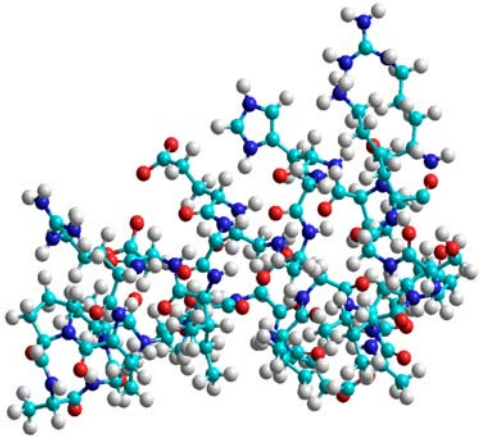
	
310 helix	Alpha helix
Energy optimization = -113.474	Energy optimization = -140.292
	
Beta turn	Left hand alpha
Energy optimization = -261.170	Energy optimization = -139.994

Figure 8 Structural representation of Pep-7 (RPHGAGEGIDRVPAGPSPSEVGLAIPSGK) contributed from constructed from standard parameters of 310 helix, left hand alpha, beta turn and alpha helix.

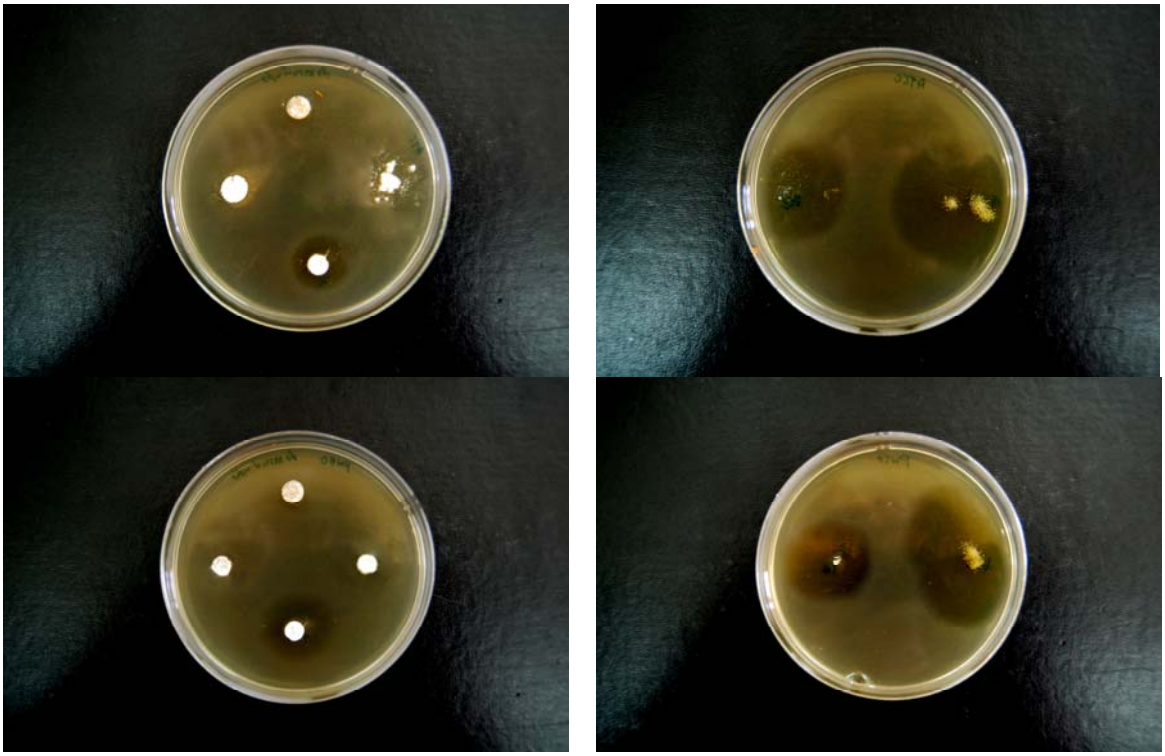


Figure 9 Clear zone obtained from Pep-7 against *P. gingivalis* at day 1 (left) and day 7 (right)

At a concentration of 5 mg per disc

ผลที่ได้จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

   K. Pangsomboon, S., Bansal, G.P., Martin, P., Suntinanalert, S., Kaewnopparat and T. Srichana. Further characterization of a bacteriocin produced by *Lactobacillus paracasei* HL32. J. Applied Microbiology, 2009, 106: 1928-1940.

2. การนำผลงานวิจัยไปใช้ประโยชน์

   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
   - เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

   พัฒนาการเรียนการสอนของปริญญาตรี-โท-เอก เพื่อทำการแยกโปรตีนบริสุทธิ์จากชีวสังเคราะห์ของแบคทีเรีย

3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

   สารสังเคราะห์เปปไทด์ที่มีฤทธิ์เฉพาะเจาะจงต่อเชื้อก่อโรคปริทันต์ เมื่อได้ดำเนินการยื่นขอสิทธิบัตรแล้วจะดำเนินการส่งต้นฉบับเพื่อตีพิมพ์ผลงานต่อไป

Pep-1

Pep-1

Pep-2

Pep-2

Pep-3

Pep-3

Pep-4

Pep-4

Pep-5

Pep-5

Pep-6

Pep-6

Pep-8

Pep-8

Pep-9

Pep-9