

CHAPTER 2

MATERIALS AND METHODS

An outlining method is presented in Diagram 2-1.

2.1 Production and isolation of bacteriocin from *L. paracasei* HL32

2.1.1 Preliminary small scale fermentations

Inoculation with 10% v/v of the bacteriocin producer *L. paracasei* HL32 incubated overnight was grown in 1 L of a Bacto™ brain heart infusion broth (BHI; Difco, USA) supplemented with 1% glucose and incubated anaerobically in a Merck® Jar system at 37 °C for 72 hours. The spent broth from the *L. paracasei* HL32 culture was centrifuged at 3000 rpm for 10 min at 4 °C (Labofuge 400R, Germany), and divided into two portions. One portion was lyophilized and the powder was used for antibacterial screening, and the other portion was used for further purification of the bacteriocin. The lyophilized powder from the spent broth is referred to Sample1 (crude).

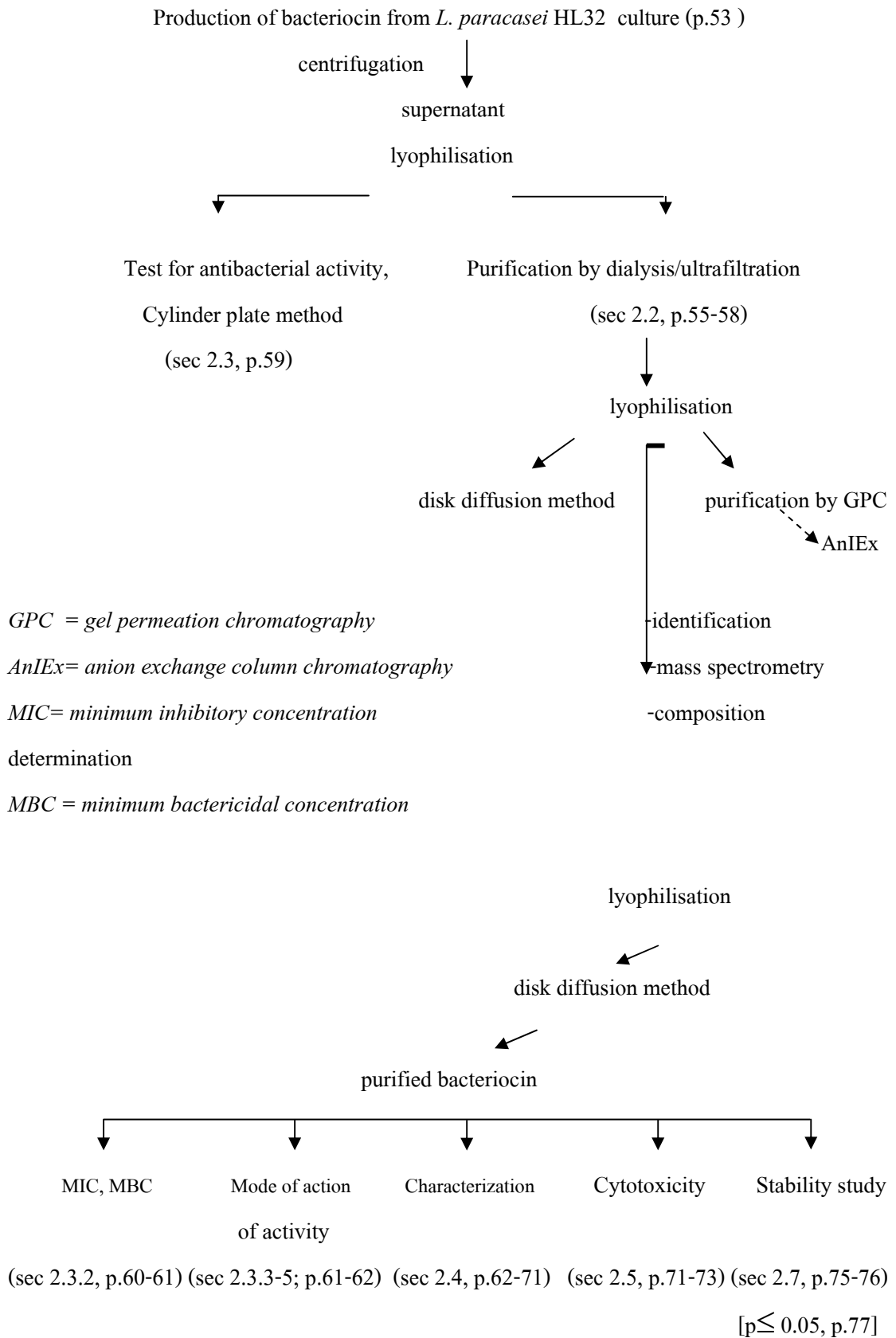


Diagram 2-1 Diagram of experimentations

2.1.2 Fermentor-scale

Bacterial fermentations were carried out in a 5 L fermentor equipped with an instrumentation for measurement and control of the same temperature at 37 °C over a period of 72 h (MBR BioReactor AG, Switzerland). The fermentor containing 3.6 L of BHI broth with an initial glucose of 1% was sterilised in situ at 121 °C for 20 min. The fermentor was inoculated aseptically with 400 mL (approximately 10%, v/v) of an exponentially growing culture. Agitation was performed at a speed of 100 rpm to maintain the fermentation broth in a homogeneous state. No aeration was performed and the dissolved oxygen during the fermentation was depleted from its initial level to that present at the end of exponential growth phase.

2.2 Purification protocol

Method of dialysis and gel permeation chromatography for bacteriocin from preliminary small scale fermentations

Step 1

Each sample of spent broth (80 mL) was dialysed using dialysis tubes with molecular weight cut-offs of 6-8 kDa (Spectra/Por[®], Spectrum Medical Industries, Inc., Texas, USA) against 2 L of 0.5 M phosphate buffer, pH 7, with continuous agitation at 150 rpm. The dialysis solution was changed every 3 h for 3 days. Temperatures were controlled at 4 °C using a two-way double jacket tank that was attached directly to a circulating water bath (compact low temperature

thermostats; Dietham, Thailand). Dialysed spent broth was lyophilised, and the powder obtained was used for antibacterial retesting and for further purification of the bacteriocin. This lyophilised powder from Step 1 (dialysis) of the purification process is subsequently referred to as Sample 2 (semi-pure).

Step 2

Sample 2 (semi-pure) was dissolved in distilled water (DW) at a concentration of 0.2 g/mL, applied to a gel-permeation column (6-cm diameter and 20-cm length, Sephadex1 G-100, Pharmacia Biotechnology, Uppsala, Sweden), and eluted with 10% aqueous methanol (J.T. Baker, USA) using a constant flow rate of 2.5 mL/min. Five millilitre fractions were collected separately up to 500 mL. The individual fractions were tested to determine a positive reaction to the ninhydrin reagent (2% w/v in DW). Every fifth fraction that gave positive was examined by thin-layer chromatography (TLC) and ultraviolet light, as described elsewhere in this chapter. The ultraviolet absorbance of each fraction was recorded between 200 and 700 nm (Spectronic Instruments Inc., USA). Finally, fractions containing the pure bacteriocin were pooled and lyophilised for further determination of antibacterial activity. Samples after gel-permeation chromatography (GPC) are subsequently referred to Sample 3 (pure).

Method of ultrafiltration, gel permeation chromatography, and anion-exchange chromatography for bacteriocin from fermentor-scale fermentations

For the fermentor-scale, the culture supernatant was concentrated to 1/10 its original volume by tangential flow ultrafiltration through a membrane (molecular cut-off 12 kDa), then was filtered through 0.45 μm acetate membrane. The filtered solution was concentrated by lyophilization and further purified by step 2. The semi-pure sample was dissolved in the eluting buffer at an initial concentration of 200 mg/mL and applied to the Superdex-G 200 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 4 mL fractions were collected sequentially and the protein content in each fraction was measured by UV absorption at 280 nm. The active fractions were pooled and the resultant solutions were dialyzed against distilled water overnight for desalting, followed by lyophilization to generate a “partially purified” sample. In due course, a portion of the sample was dissolved (100 mg/mL) in 20 mM Tris-HCl buffer (pH 8). The partially purified sample was further 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 of NaCl upto 0.5 M (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 which contained inhibitory activity were eluted 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 (2x5 cm) (HiTrap Q HP, Amersham, Pharmacia Biotech) and the final active solid obtained by lyophilization. This lyophilised powder from Step 2 (column chromatography) of the purification process is subsequently referred to as Sample 3 (pure). Protein content (in mg per mL), was estimated by the Lowry method (Lowry *et al.*, 1951), using human albumin as a standard.

2.3 Antibacterial activity tests

2.3.1 Bacterial strains and growth conditions

P. gingivalis ATCC 33277TM, *P. gingivalis* ATCC 53978TM, *P. intermedia* ATCC 25611TM, *Tannerella forsythensis* 43037TM, *Streptococcus sanguinis* 10566TM, *Streptococcus salivarius* 25975TM, *S. aureus* ATCC 25923TM and *Escherichia coli* ATCC 25922TM were used in this study. *S. aureus* and *E. coli* represented Gram-positive and Gram-negative bacteria, respectively, and were used as reference strains for antibacterial activity testing. The selection of the indicator strains (Gram-positive bacteria: *S. sanguinis* and *S. salivarius*; Gram negative: *T. forsythensis*, and *P. intermedia*) was based on their importance in the oral ecosystem. The bacteria were grown under the conditions specified in Table 2-1.

To standardize the cells, the reference strains were grown to reach log phase and then the suspension was adjusted to 25% transmittance at an OD560 corresponding to approximately 10^8 colony-forming units/mL, and this was further used for antibacterial activity testing.

Table 2-1 Bacterial strains and growth conditions

Organism	ATCC number	Media		Conditions	
		Broth	Agar	Temperature	Atmosphere
<i>Porphyromonas gingivalis</i>	33277 TM	sBHI	TSA		
<i>Porphyromonas gingivalis</i>	53978 TM	(BHI with YE 5 mg/mL,	with YE 1 mg/mL,		Anaerobic gas mixture,
<i>Prevotella intermedia</i>	25611 TM	vitamin K1 5 µg/mL and	vitamin K1 5 µg/mL,		80%N ₂ -10%CO ₂ -10%H ₂
<i>Tannerella forsythensis</i>	43037 TM	hemin 5 µg/mL)	hemin 5 µg/mL and		
			5% blood		
<i>Streptococcus sanguinis</i>	10556 TM	BHI	TSA with 5% blood	37°C	5% CO ₂
<i>Streptococcus salivarius</i>	25975 TM	BHI	BHI agar		
<i>Staphylococcus aureus</i>	25923 TM	Mueller-Hinton broth	Mueller-Hinton agar		Aerobic
<i>Escherichia coli</i>	25922 TM	Mueller-Hinton broth	Mueller-Hinton agar		

sBHI: supplemented BHI; BHI: brain heart infusion broth; TSA: tryptic soy agar; YE: yeast extract

2.3.2 Susceptibility tests

Antibacterial activity was screened by the cylinder plate method (Koo *et al.*, 2000). These tests were carried out six times for each strain. Briefly, plates containing sBA or MHA agar were brushed using sterile swabs with 25% transmittance test solitary organisms (*P. gingivalis*, *S. aureus* and *E. coli*). Solutions of Samples 1-3 of the lyophilised powders were prepared at a concentration of 20 mg/300 mL DW. Solutions of the reference antibiotics were prepared at a concentration of 30 µg/300 mL DW. These samples were sterilized by filtration through wetting-agent-free cellulose acetate 0.2-mm filter (Sartorius, Germany). Three hundred microlitres of reference antibiotic solution or 300 µL of the test sample solutions were placed in the cup on the

agar plate. *P. gingivalis* on sBA was incubated in an anaerobic glove box (Thermo Forma, Germany) with 80% N₂, 10% CO₂ and 10% H₂ at 37 °C for 72 h, whereas *S. aureus* and *E. coli* on MHA were incubated in an aerobic incubator (Heraeus B5060 E, Germany) at 37 °C for 24 h. After the incubation period, the diameter of the inhibition zone was measured with an antibiotic zone reader (Fisher Scientific™, USA) and recorded in millimetres. Sample 3 (pure) was selected for further testing of the minimum inhibitory concentration (MIC).

MICs were determined in a microtitre assay (Javadpour *et al.*, 1996) by inoculation of 100 µL of *P. gingivalis* suspended in supplemented BHI (sBHI; yeast extract 5 mg/mL, vitamin K₁ 5 mg/mL and hemin 5 mg/mL, final concentration 5 x 10⁵ colony-forming units/mL) in a 96-well microtitre tray with two-fold serial dilutions by adding 100 µL of a solution of Sample 3 (pure) powder or control antimicrobial agents (tetracycline, metronidazole). The final concentrations of the test powder sample were 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0.0625 mg/mL, and for the antimicrobials were 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.0156 µg/mL (positive controls). The microbial suspension added with water served as a negative control. The plates were incubated anaerobically for 72 h. The MICs were recorded as the lowest concentration for 90% inhibition. Minimum bactericidal concentrations (MBCs) were determined by culturing on sBA for 48 h. The percentage of inhibition is expressed by the following equation:

$$\% \text{inhibition} = \frac{\text{OD560 in control group} - \text{OD560 in tested group}}{\text{OD560 in control group}} \times 100$$

OD560 in control group

For studies of the kinetic death rate of Sample 3 (pure), a cellular suspension (100 mL, 10^5 colonyforming units/mL) of *P. gingivalis* was inoculated into sBHI. The test powders (aqueous solutions, final concentration 2-8 mg/mL) were added, and the mixtures were incubated at 37 °C for 0, 2, 5, 9, 24, 48 and 72 h. At designated intervals, 100- μ L portions were taken, diluted and dropped on to sBA, the plate was incubated for 48 h, and colonies were counted.

2.3.3 Scanning electron microscopy

Scanning electron microscopy (SEM) was employed to observe bacterial surfaces. One-millilitre aliquots of each of the three test bacterial suspensions were pooled from the 72-h cultures, and treated with each test agent [Sample 3 (pure), tetracycline, metronidazole or 0.3 M lactic acid, pH 4]. After 72 h of contact with the samples, the cell suspension was filtered using a 0.22- μ m cellulose acetate membrane filter, and immediately fixed with 2.5% glutaraldehyde in phosphate buffer saline. After gold coating, the cells were viewed under a scanning electron microscope (JSM-5800 LV, JEOL, Japan).

2.3.4 Transmission electron microscopy

The effect of bacteriocin on the intracellular structure of *P. gingivalis* was assessed using transmission electron microscopy (TEM). *P. gingivalis* ATCC 33277 and *P. gingivalis* WP 50 grown in supplemented BHI broth were treated with the minimum inhibitory concentration (MIC)

of bacteriocin for 2 h. The bacteria were isolated by centrifuging the cultured cells at 10000 g for 5 min. Samples of harvested sedimented cells were prepared for TEM by fixing with 2.5% (v/v) glutaraldehyde, post fixing with 1% (v/v) osmium tetroxide for 1 h and then dehydrating with ethyl alcohol. The sections were embedded in epoxy resin and cut, and then 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).

2.3.5 Potassium leakage

P. gingivalis was grown in supplemented BHI broth in the presence of bacteriocin at the MIC. At periodic time interval of 3, 4, and 6 h, the broth was removed and centrifuged at 3000 RPM 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. The untreated *P. gingivalis* and sBHI broth served as a positive and negative control.

2.4 Characterisation and identification of the bacteriocin

The bacteriocin was characterized and analysed by UV-spectroscopy, RP-HPLC, mass spectrometry and amino acid analyzer, and protein sequencing (Walker, 1994; Javadpour *et al.*, 1996; Ryadnov *et al.*, 2002; Kondejewskii *et al.*, 1996; Lesk, 2004). The determination of suitable adsorption wavelengths was carried out with UV spectrophotometer (Spectronic Instruments, Inc. (USA)) (Copeland, 1994); the molecular weight was identified by ESI-MS (electrospray ionisation mass-spectrometry). Additionally, the sequence of peptide was analyzed by converting amino acid chains to the phenylthiohydantoin (PTH) derivative using Edman degradation sequence analysis (Walker, 1994).

2.4.1 UV study of the peptide/ amino acids

A suitable adsorption wavelength of the bacteriocin for was monitored for quality control purposes as following (Copeland, 1994):

- 1) Add to a clean, dry, quartz cuvette enough buffer (CE) or solvent (HPLC) to fill the cuvette at least three-quarters full volume.
- 2) Record the absorption spectrum between 200 and 400 nm, and store this spectrum as a reference or baseline spectrum.
- 3) Remove the buffer, clean and dry the cuvette.
- 4) Filter a sample through a 0.45 μm filter.

5) Fill the cuvette with sample of amino acid or peptide, and record the spectra as above condition.

2.4.2 Ninhydrin test (Qualitative colour methods)

The ninhydrin test was used for free N^α-amino and –imino groups determination. Isolated and purified samples were placed in a small test tube and 2-3 drops of a reagent (0.2% w/v in water) was added. The tube was heated at 100 °C for 5 min and viewed against a white background. A negative test (no primary amines) was indicated by a straw yellow colored solution. A positive control sample (primary amines) gave a strong dark blue/ purple (McGinn, 1996).

2.4.3 Thin-layer chromatography

Silica gel G60 F₂₅₄ alumina backed plates (Merck[®], Germany) (4 cm x 8 cm) were used in ascending technique. Samples 1 (crude), 2 (semi-pure) and 3 (pure) powders were dissolved in DW to a concentration of 1 mg/mL, and 10 mL of each was applied to TLC plates. The plates were developed with either n-butanol/acetic acid/water (J.T. Baker, USA) : 5/3/1 (v/v/v), or chloroform, methanol and 0.1% trifluoroacetic acid in water (170:120:15 v/v/v) using the chromatography tank. Separation took place in a glass tank, which contained the developing solvent to a depth of about 0.5 cm. This system was allowed at least 30 minutes to ensure that the atmosphere within the tank became saturated with solvent vapor (equilibration). Notably, the system must be the closed system. All TLC plates were run in triplicate. The ultraviolet

absorbing spots were detected at 214 and 254 nm. The plates were then sprayed with ninhydrin (0.1% w/v in water-saturated n-butanol), and heat at about 90 °C for 5-10 min to detect compounds. Determination of the compound will be carried out on the paper when it formed a purple colored spot. The ratio of the distance moved by a compound to that moved by solvent is known as the R_f values and is a constant for a particular compound using equation 1.

- 1) Spot approximately 1 μ L of the sample solution on each of TLC plates.
- 2) Allow 15 to 20 minutes air drying to ensure complete evaporation of the spotting solvents.
- 3) Develop the chromatogram sheets in the two developing solvents for a distance of 8 cm.
- 4) Dry the developed TLC plates and spray with the ninhydrin solution.
- 5) Heat gently for several minutes until separated spots was clearly visible.

The visualization with ninhydrin has limits of detection that may vary from 0.01 to 0.5 μ g/mL, depending on the particular amino acids as well as the method of separation employed. Therefore, TLC densitometry was developed; the chromatogram was recorded at either 220 or 280 nm.

$$R_f = \frac{\text{distance traveled by center of component}}{\text{distance traveled by solvent}} \quad \text{equation 1}$$

2.4.4 RP-HPLC

The RP-HPLC conditions were a 5- μ m particle size Waters Spherisorb[®] C₁₈ column, bonded silica, 300 Å pore size, 4.6x250 mm; Water[™] 717 plus autosampler, Water[™] 600 controller,

Water™ 600 pump, Water™ 996 Photodiode Array Detector (USA) using a Software Millennium version 2.1. The elution conditions employed for RP-HPLC were as following: solvent system, 0.1% aqueous TFA (A solution) and acetonitrile containing 20% A solution (B solution); flow rate, 1 mL/min; temperature, 25 °C; and UV detection, 220 nm and 280 nm. Elution was carried out with 5% B solution for the first 5 min and then with a linear concentration gradient of B solution, 20–60% for 40 min. Retention times of peptides were determined by RP-HPLC. and monitored at 220 nm.

- 1) Install Waters Spherisorb® column (C₁₈ bonded silica, 300 Å pore size, 5 µm, 250 mm length, 4.6 mm id, Ireland).
- 2) Equilibrate the column by pumping eluent at 1 mL/min (diameter of 4.6 mm) for at least 150 mL. Monitor detector baseline (214 nm) until equilibrium and then zero the detector. Inject 5 µL of HPLC quality water and run a blank gradient to identify any system peaks and to balance baseline (TFA), if necessary. The HPLC eluents were either used immediately after preparation or stored at –20 °C, filtered pass trough 0.45 µm and degassed with helium before daily usage.
- 3) After activating a C-18 bonded-phase silica-based column by sequentially passing methanol, water and mobile phase through it. One should wait until the absorbance returns to baseline (usually 15-20 min) before initiating the elution. The sample solution (100 µL) was injected automatically into the column by autosampler.

2.4.5 Sodium-dodecyl sulphate-polyacrylamide gel electrophoresis

Molecular mass determination was subsequently performed on Sample 3 (pure) by sodium-dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrospray ionisation mass spectrometry (ESI-MS). The bacteriocin was tested using SDS-PAGE on 15% gels. Samples were dissolved in DW at a concentration of 6 mg/100 mL plus loading dye with an equal volume of buffer (100 mmol/L Tris, pH 6.0, 200 mmol/L dithiothreitol, 200 mL/L glycerol, 2 g/L bromophenol blue and 40 g/L sodium-dodecyl sulphate). These samples were heated at 100 °C for 3 min, and 15 mL of each was applied to a separating gel. Gel electrophoresis was carried out at a constant voltage of 100 V at an ambient temperature for 60 min or until the tracker dye reached the front of the gel. The gel was stained with Coomassie blue G-250 for 12 h, and then fixed in 20% ethanol: 10% glacial acetic acid in water for 30 min at room temperature. A protein marker solution, being a broad reagent (2-212 kDa) (BioLabs, New England, USA), was used to estimate the molecular weight of the bacteriocin.

In addition, the purity of each of the sample from the fermentation-scale, 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 (10 x Tris/Glycine/SDS buffer, Bio-Rad). After electrophoresis, the gels were immediately rinsed with distilled water for 3 times, then placed into GelCode[®] protein stain solution (Pierce, USA) for 1 h with gentle agitation, and then followed with distilled water until background was clear. For SYPRO Ruby staining, after results of the former GelCode[®] were

recorded, then the gels were washed further with distilled water over night, and then soaked directly into the SYPRO ruby stain solution over night with gentle agitation. Later on, the gels were destained in 10% methanol/ 7% acetic acid for 1 h (replacing the wash solution after 30 minutes) and given a final rinse with distilled water for 3 times prior to image acquisition. At the same day as the SYPRO Ruby gel were destained, they were visualized using a UV transilluminator, then took a photo. Prestained SDS-PAGE Standards, which spanned a broad range of MW (7.4-192 kDa, Bio-Rad), were used as molecular weight markers.

2.4.6 electrospray ionization mass spectrometry

ESI-MS was used to analyse the molecular ions of the bacteriocin. The bacteriocin was dissolved in 10% methanol at a concentration of approximately 5 pmol/mL. Ten microlitres were injected into the direct probe of the MS, and the solvent (10% methanol) was evaporated. The nozzle voltage was increased from 70 to 3500 V to cause fragmentation and nebulised with nitrogen. During the ESI-MS operation, the hexapole was typically scanned from m/z 100 to 4,000 at 2 s/scan. Calibration was performed by separate injection of horse heart myoglobin (16950.4 Da).

2.4.7 Amino acid analysis

For determination of amino acid composition, a sample (500 mg) was hydrolysed in 6 N HCl and placed in a heating block at 110 °C for 22 h to yield the amino acids in free form (Liu *et al.*, 1995)

The free amino acids were derivatised with an AccQ (6-aminoquinolyl-N-hydroxysuccinimidyl

carbamate) fluor derivatisation buffer and AccQ fluor reagent, and heated at 55 °C for 10 min on a heating block. The amino acid analysis was carried out on a Waters Alliance 2695 amino acid analyser equipped with a heater that was connected to a multiwavelength fluorescence detector.

2.4.8 Deglycosylation of the bacteriocin

Deglycosylation was performed to determine the carbohydrate part of the bacteriocin. Ten milligrams of Sample 3 (pure) powder were hydrolysed with 100 mU/mL amyloglucosidase (Sigma, Switzerland) in 3 mL of 50 mM citrate buffer, pH 4.5, for 3 min at 55 °C. The resultant sample was examined by Benedict's sugar test and Seliwanoff's resorcinol test for detection of reducing sugars and furfural substances, respectively. Cleaved protein was isolated continuously by GPC. The bacteriocin was analysed by ESI-MS before and after deglycosylation.

2.4.9 Protein sequencing

2.4.9.1 N-terminal sequencing

After deglycosylation, a sequence of 15 amino acids was determined from the N-terminus by Edman degradation with an applied Biosystem model Procise 492 HT automatic sequencer (Applied Biosystem). PTH-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>).

2.4.9.2 MALDI-TOF MS/MS

1) In-gel reduction/alkylation

2) In-gel tryptic digestion/ extraction/desalting

3) MALDI ToF-ToF MS and MS/MS with database searching

The pure sample which was run on 12.5% SDS-PAGE and 56 kDa band was excised from the gel. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was done at Protein and Proteomic center, Department of Biological Sciences, National University of Singapore. The protein identification involves using tryptic digestion to obtain masses of individual peptides derived from the protein. Mass spectra of peptides were measured on a mass spectrometer with the method of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy. The masses were obtained from fragmentation of occurring peptides were compared to deposit sequences against an online database 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 are used to determine the closest protein matches.

2.4.10 CD spectroscopy

The circular dichroism (CD) spectra were determined with an Applied Photophysics Ltd Chirascan spectrometer. The experiments were carried out in the wavelength region 400-230 nm (near ultraviolet) and 260-180 nm (far ultraviolet) at 0.5 nm in a cuvette with 0.1-cm and 0.05 cm Supracil rectangular cells, respectively. The scanning speed was 20 nm/min and 100 nm/min for near UV and far UV, correspondingly with a band width of 1.0 nm. The experiment was run continuously with pure evaporated nitrogen throughout. Secondary structure prediction of the bacteriocin sequence (Zdobnov and Apweiler, 2001) were also accomplished using the program available through internet (<http://swissmodel.expasy.org>)

2.5 Cytotoxicity

2.5.1 Hemolysis assay

Cytotoxicity was determined by assessing the hemolytic activity using modified published methods (Kondejewski *et al.*, 1996). In brief serial dilutions of the bacteriocin contained in 100 µl phosphate buffer saline (PBS, 0.01 M) at pH 7.4 were incubated with 50 µl of 1/25 packed volume of human red blood cells distributed in microtiter wells plate. 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 2.

$$\% \text{hemolysis} = \frac{(A_{\text{exper}} - A_{\text{control}})}{(A_{\text{total}} - A_{\text{control}})} \times 100 \quad \text{Equation 2}$$

where **A_{exper}** is the absorbance values of supernatants from treated red blood cells

A_{control} is the absorbance values of supernatant from non-treated red blood cells

A_{total} is the absorbance of the red cells treated with 0.1% Triton X-100, corresponding to 100% lysis

To prepare the red blood cells, the packed volume of cells were prepared by centrifugation about 2 mL heparinized human blood at 3000 rpm for 10 min at 4 °C to remove the buffy coat, and the remainder was washed with 2 mL PBS pH 7.4 and centrifuged several times until a clear supernatant was obtained. Cells count of the washing packed red cells was performed using hemocytometer (Improved Neubauer Brightline, Hausser Scientific, Boeco, Germany).

To determine the percentage of hemolysis, the concentration of bacteriocin in the PBS using 500 µl volumes was incubated with 250 µL of 1/25 packed volume of cells in Eppendorf tube. After 48 h incubation at 37°C in a thermal shaker with a gentle mixing, at 1, 6, 12, 24, 48 and 72 h, the tubes were centrifuged at 4000 rpm for 10 min. Following centrifugation the hemoglobin concentration in the supernatants was transferred to microtiter plates for 150 µL in each well. Optical density readings were measured at 540 nm of the absorption intensity mode (hemoglobin absorption maxima=542 nm), and the percentage of hemolysis was calculated with the following equation 2, where **A_{exper}** and **A_{control}** represent the absorbance values of supernatants from treated and non-treated red cells, and **A_{total}** is the supernatant of red cells treated with 0.1% Triton X-100 which represent the 100% lysis. The bee venom mellitin

solutions in PBS (concentrations: 0.125, 0.25, 0.5, 1.0 and 2.0 μM) and PBS were also as a positive control and negative control, respectively. Both the positive and negative controls were prepared and incubated in a same manner as the experimental specimens.

2.5.2 Mammalian Cell Assays

The toxicity of peptides to induce cell death of either human fibroblasts or human periodontal cell ligament (PDL) (gifted from Kamolmattayakul, S. and Wattanaarunwong, N., Prince of Songkla University) was determined by 2-fold serial dilution assay; 50 μL of a stock peptide solution was diluted with an equal volume of Minimum Essential Medium (MEM), and 1:2 serial dilutions in MEM are prepared. Each dilution was applied to a 1-day old monolayer of the cells (approximately 1×10^4 cells/well) maintained in a 96-well plate with fresh MEM (50 μL /well). Bacteriocin-treated and negative control (no bacteriocin) cells were incubated at, 37°C for 30 min. The bee venom mellitin solutions in PBS served as a positive control. The supernatant is 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. A sublethal dose was defined as a highest dilution in which only 1-10 adherent cells were not stained. No cytotoxicity means that cell population survival is unchanged from negative controls (Javadpour *et al.*, 1996).

2.6 Collection of biological sample

2.6.1 Subjects

The study group were composed of 12 chronic periodontitis patients (age 45 ± 4.2 years; mean \pm SD) and 15 periodontally healthy subjects (age 38 ± 3.5 years; mean \pm SD). Both groups had no systemic diseases, were not receiving antibiotic medication, and had received no 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 so designated if there was no evidence of a periodontal pocket or attachment loss and a gingival index score 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.

2.6.2 Collecting saliva and crevicular fluid

Saliva was collected between 9:00 a.m. and 1:00 p.m. Unstimulated saliva was collected by spitting method with slight modification as previously described (Navazesh and Christensen, 1982). Briefly, subjects were instructed to tilt their heads forward, keep their eyes open, and make as few movements as possible, including swallowing. The subjects were instructed to swallow to begin a collection trial, and were then told to allow saliva to spit out behind closed lips into plastic cup and expectorated at the end of each min of a test trial for 5 min. Thereafter, paraffin-stimulated whole saliva was collected from the volunteers, using the method as described

previously (Johansson *et al.*, 1994), the subjects were instructed to chew a 1-g piece of paraffin to softness, swallow the secreted saliva, and then start to chew for 1 min and spit the secreted saliva into plastic cup for 5 min as previously described (Johansson *et al.*, 1994). The unstimulated and paraffin-stimulated whole saliva were stored at -20 °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 (Griffiths *et al.*, 1988). Prior to sampling, the area was isolated with cotton rolls, and the supragingival region of the tooth surface was cleaned and dried with the sterile cotton buds. After leaving the paper strips at each site for 1 min, each strip was transferred to an Eppendorf vial containing 1 mL of sterile distilled water. The vials were stored at -20 °C until further investigation.

2.7 Stability studies

2.7.1 Stability of bacteriocin in biological fluids

Saliva and gingival crevicular fluid (GCF) were sterilized by filtration through 0.22 µm cellulose acetate membrane filter (Sartorius, Germany). The isolated bacteriocin was dissolved in either fluid at the level of the MIC. The % inhibition of *P. gingivalis* ATCC 33277 was determined using the microtiter assay for the antimicrobial activity, as described previously (in section 4.3.2). The bacteriocin dissolved in water and water served as a positive control and negative control, respectively.

2.7.2 Thermal stability

The purified bacteriocin (0.14 mM) was redissolved with sterilized water and heated in a boiling water baths adjusted to temperatures of 25 °C (control), 50 °C, 80 °C and 100 °C. Samples were removed after 5, 10, 15, 20, 25, 30, and 60 min. The purified bacteriocin (0.14 mM) was also autoclaved at either 100 °C for 30 min, 110 °C for 30 min, or 121 °C for 15 min for autoclaving. After heating treatment, the samples were cooled to room temperature and tested for antibacterial activity by the microtiter assay (in section 2.3.2). The bacteriocin dissolved without any thermal treatment and water served as a positive control and negative control, respectively.

2.7.3 pH stability

To determine the effect of pH on the bacteriocin activity, the purified product (0.14 mM) was redissolved in either 5 mM phosphate-citrate buffer (pH 2.6-7.4) or 5 mM Tris-HCl pH (8.0-9.0) and incubated at room temperature for 1 h. Bacteriocin dissolved in water (pH 6.8 ± 0.2) served as positive controls. The microbial suspension added with water served as a negative control. The antibacterial activity was subsequently determined using the cylinder plate method (in section 2.3.2).

2.7.4 Stability to enzymes

The purified (0.28 mM) bacteriocin was dissolved in 500 μ l of distilled water, and the solutions were incubated either with 500 μ l α -chymotrypsin (500 μ g/mL in 0.01 M phosphate buffer, pH 7.8), trypsin (500 μ g/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 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 then was filter sterilized. The untreated bacteriocin and distilled water served as a positive control and negative control, respectively. The antibacterial activity was determined using the cylinder plate method, as described previously (in section 3.4.2).

2.7.5 Storage stability

Accurately purified bacteriocin samples (32 mg) were stored in sealed amber bottle as a solid and incubated in desiccator at 4 °C, room temperature (25 °C) or at 37 °C. A series of samples were prepared at time zero and stored at the separate temperatures with individual bottled samples being withdrawn periodically up to 3 months. The antibacterial activity was determined using the microtiter assay (in section 3.4.2).

2.8 Statistical method

The differences in the % inhibition after the exposure of bacteriocin to a different heat treatment were 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.