CHAPTER 3

RESULTS & DISCUSSIONS

OBJECTIVE 1 to purify antimicrobial protein(s) from *Lactobacillus paracasei* HL32

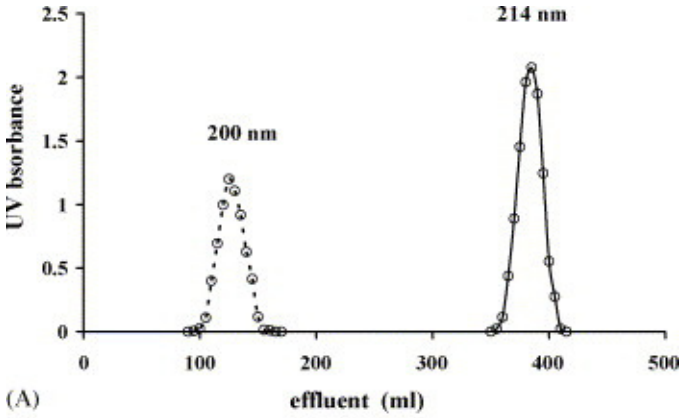
RESULTS

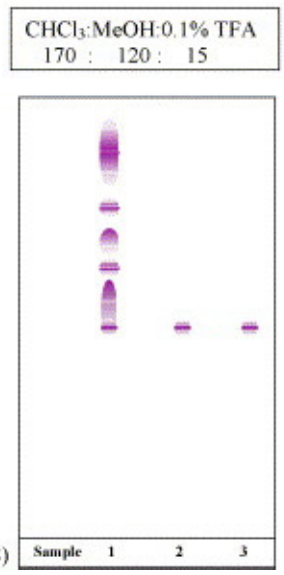
Production of bacteriocin

During the fermentation without pH control, the pH at the beginning was about pH 7.2, but the pH at the end of the preliminary small scale and fermentor-scale fermentations respectively decreased to about pH 5.43±0.13, and pH 3.87. In comparison, each spent broth sample (80 ml) of *L. paracasei* HL32 culture for preliminary small scale fermentations gave lesser bacteriocin than fermentor-scale fermentations about 2.27 g powder and 2.65 g of crude powder (Sample 1). After purification with dialysis we obtained 0.66 g and 0.83 g of semi-pure powder (Sample 2). At final column purification 0.38 g powder and 0.48 g pure powder (Sample 3), respectively after lyophilization.

Purification of the bacteriocin

On GPC of Sample 2 (semi-pure), the positive fractions to ninhydrin were collected from fractions 71–82 (see Fig. 3-1(A)) and these combined fractions gave powder after lyophilisation. The maximum absorbance of fractions 71–82 was at 214 nm and low at 254 nm (see Fig. 3-2(A)).





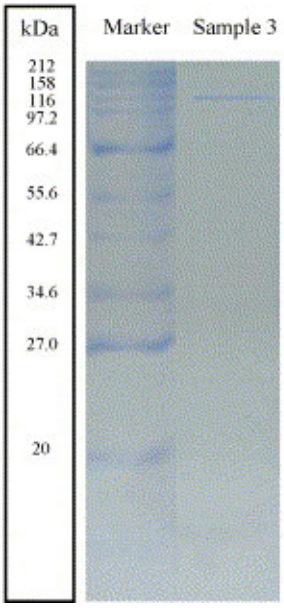
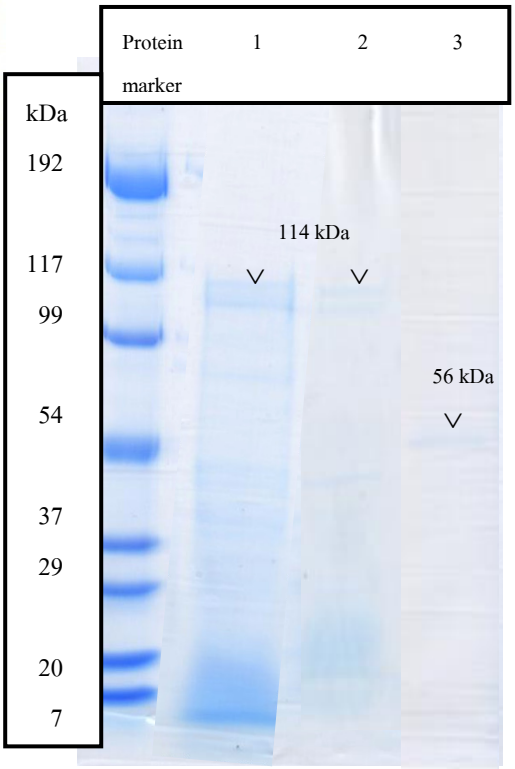


Figure 3-1 Elution trace profile from gel-permeation chromatography, thin-layer chromatography and sodium-dodecyl sulphate-polyacrylamide gel electrophoresis of each sample from purification steps of *L. paracasei* HL32. Sample 1 (crude), Sample 2 (semi-pure) and Sample 3 (pure). (A) Sephadex G-100 with 10% aqueous MeOH, the ultraviolet (UV) absorbance of each 5-ml fraction was recorded. (B) Silica gel plates were developed with the mobile phase showing a single ninhydrin-positive spot for Samples 2 and 3 after purification steps (Rf 0.6). (C) Gel stained with Coomassie blue G-250; protein marker (broad reagent) (left lane); Sample 3 (right lane). **(D)** SDS-PAGE of bacteriocin produced by *L. paracasei* HL32 on 4-15% Tris-HCl gel; Sample 2 (Lane1), Sample 3 (Lane 2) and Non-binding product obtained from AnIEx (Lane 3).



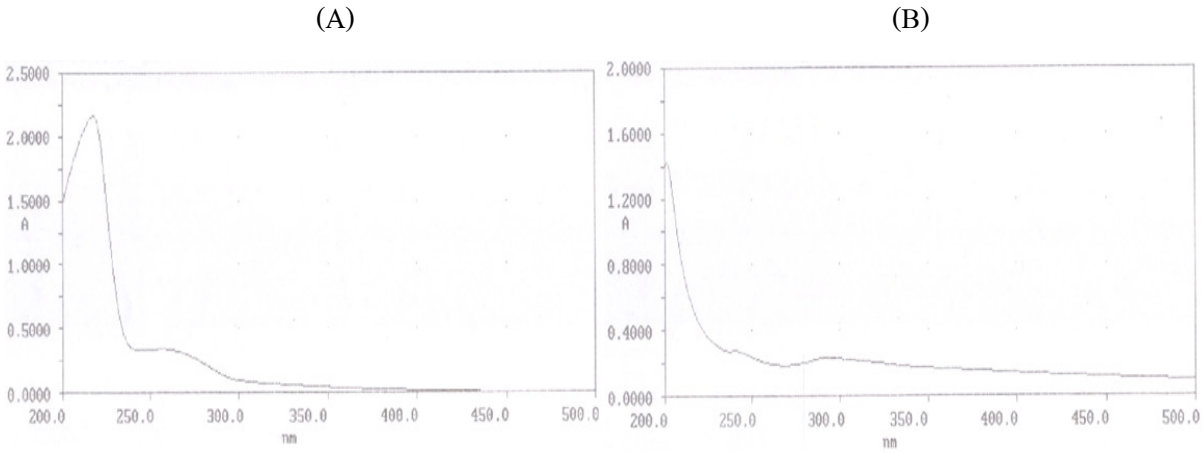


Figure 3-2 UV study, (A) Sample 3 (pure) 2 mg/ml; (B) impurity protein 5 mg/ml

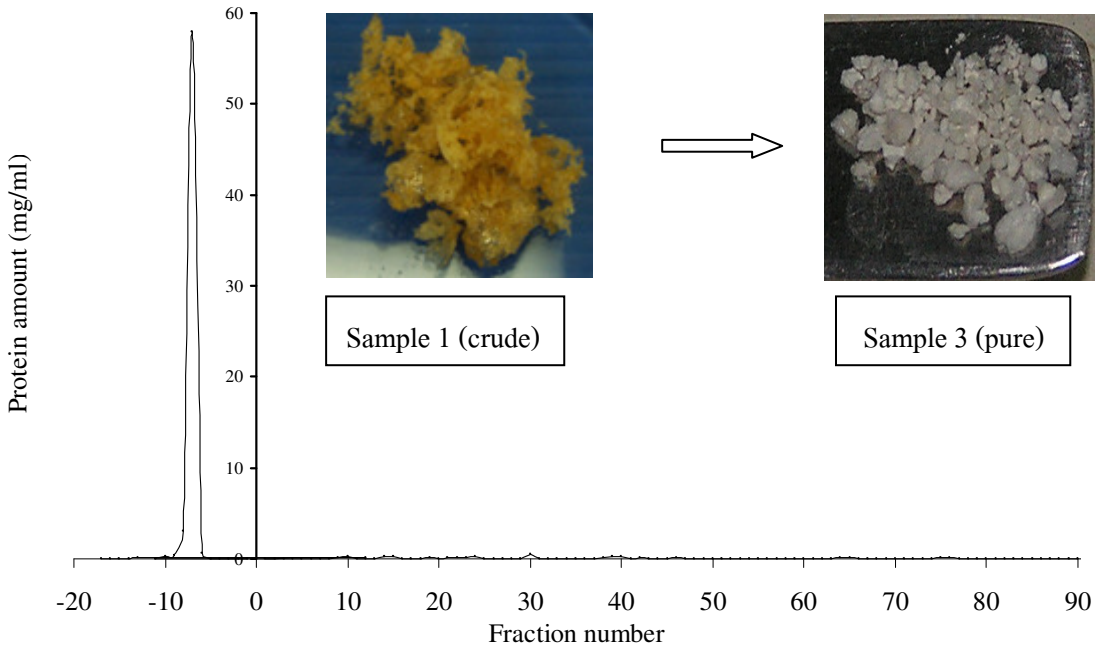


Figure 3-3 Partially purified bacteriocin on anion exchange resin (HiTrap Q HP, Amersham). None binding fractions and binding fraction were pooled separately for further characterization

The elution trace profile from GPC shows that Sample 3 (pure) was separated from impurities in fractions 19–33 (unidentified impurities), and these impurities showed high absorbance at 200 nm and no absorbance at either 214 or 254 nm (see Fig.3-2(B)). Anion and cation exchange resin did not retain the bacteriocin. When the major product was applied to a column of anion and cation exchange, it was found to pass through the column without absorbing on the column; the bacteriocin was recovered in the flowthrough step. However, traces (yellowish substances) were retained on anion exchange column; they were flushed out in the eluent volume (see Fig.3-3). Afer this step, bactericoin become white powder (see Fig.3-3).

DISCUSSIONS

The purification steps resulted in a yield of about 16% and 18% pure bacteriocin for preliminary small scale fermentations and fermentor-scale, respectively (0.38 g and 0.48 g of pure bacteriocin from 2.27 g and 2.65 g of crude lyophilisation of spent broth). Both %yields in the first and second experimental protocols gave higher than other studies (Mortvedt *et al*., 1991; Kabuki et al., 1997). There are differences in the results of the purification of bacteriocin and the yield in the first and second protocols. Using a simple growth medium containing with glucose, it was possible to obtain a production of bacteriocin from *L. paracasei* HL32. Quality control of the proteinatious fractions after purification was carried out. The maximum absorbance peak of purified protein at 214 nm seems to be peptide bonds (Fig.3-1(A)); however, the peak at 254 nm

is not a typical protein absorbance peak (Walker, 1994). The results from the step 1 purification indicate that the bacteriocin is an extracellular antagonistic molecule with a molecular weight greater than the molecular cut-off of the dialysis tubes (6–8 kDa). As the pH of the aqueous solutions of these samples increased from 3.83 to 7.04 after step 2 purification (see Table 3-2) were observed; these phenomena were presumably related to lactic acid and propionic acid completely elimination.

Comparison of the difference methods in step 1 purification revealed that separation by ultrafiltration is less expensive and more rapid than dialysis. Furthermore, lactic acid and propionic acid were completely removed after ultrafiltration method.

At step 2, impurities were removed (see Fig.3-1(A)), after that the purity of the bacteriocin increased dramatically. These results indicate that the purification steps were successful.

OBJECTIVE 2 to study antibacterial activity of bacteriocin from *L. paracasei* HL32 against *P. gingivalis*, and also compared to effects of tetracycline and metronidazole

RESULTS

Antibacterial activity and mode of action

Antibacterial activities are summarized in Table 3-1. Briefly, Sample 1 (crude) powder had a growth inhibition zone on agar with all three strains. Interestingly, tetracycline gave similar zones of inhibition to crude sample (sample 1) with respect to *E. coli* and *S. aureus* (16.4 and 23.7 mm for tetracycline, 18.2 and 18.4 mm). However, with *P. gingivalis*, tetracycline was considerably more potent [45 mm for tetracycline, 11.7 mm for Sample 1 (crude)]. Step 1 purification (dialysis) and Step 2 purification (GPC) appeared to remove the substances having antimicrobial activity against *E. coli* and *S. aureus* in the crude spent broth. Samples 2 and 3 did not show a zone of inhibition against either of these two bacterial strains. However, antibacterial activity against *P. gingivalis* was maintained and the zone of inhibition against this strain of bacteria increased with purification (11.7, 16.0 and 27.0 mm for Samples 1, 2 and 3, respectively). The antibacterial profiles of Samples 2 and 3 were similar to that of metronidazole, i.e. no activity against *E. coli* and *S. aureus*, but active against *P. gingivalis*. The *E. coli* and *P. gingivalis* were susceptible to tetracycline with MICs of 0.2–2 μM and MBCs of 1–8 μM. *P. gingivalis* was

susceptible to metronidazole with an MIC of 0.7 μM and an MBC of 1.4 μM, whereas Sample 3 (pure) powder had MIC and MBC values of 0.14 mM.

A crude product (Sample 1) isolated from the supernatant obtained from bacterial cultures of *L. paracasei* HL32 has been found to inhibit a range of selected microorganisms, but a narrower spectrum of the activity was demonstrated after the elimination of organic acids. Lactic acid, a major by-product of lactobacilli fermentation, was removed by dialysis and the purification procedures employed in this study lead to the isolation of a purified bacteriocin.

The activity spectrum of purified bacteriocin against oral bacterial species is shown in Table 3-2. Both strains of *P. gingivalis* were susceptible to the antimicrobial action of bacteriocin (MIC: 0.14 mM), whereas the other oral pathogens and normal flora were not susceptible to the bacteriocin, except *P. intermedia* showed some resistance to bacteriocin. The antibacterial profile of bacteriocin showed selectivity against *P. gingivalis* whereas metronidazole established a narrow spectrum of activity against *P. gingivalis, P. intermedia* and *T. forsythensis*.

The kinetic death rate of *P. gingivalis* when challenged with different agents is shown in Fig.3-4. At lower concentrations of Sample 3 (pure) (0.035–0.07 mM), the growth appeared to decline very slightly for 2 h followed by incrementation back to the normal growth rate thereafter. At the critical concentration (0.14 mM) during incubation times of 10 min, 1 h and 2 h, the numbers of *P. gingivalis* decreased dramatically with no viable counts after 2 h.

Table 3-1 Antibacterial screening of biosynthetic substance(s) of *L. paracasei* HL32 culture, tetracycline, and metronidazole against several bacterial strains

Agent	pH (± SD)	Bacterial strains	Amount / inhibition zone	
			n=3	n (mm±SD)
Sample 1 (crude)	3.83 (± 0.5)	*E. coli*, ATCC 25922	20 mg	18.2 (0.4)
		S. aureus, ATCC 25323	20 mg	18.4 (0.3)
		P. gingivalis, ATCC 33277	20 mg	11.7 (0.7)
Sample 2 (semi-pure)	6.66 (± 0.2)	*E. coli*, ATCC 25922	20 mg	(-)
		S. aureus, ATCC 25323	20 mg	R
		P. gingivalis, ATCC 33277	20 mg	16.0 (0.4)
Sample 3 (pure)	7.04 (± 0.1)	*E. coli,* ATCC 25922	20 mg	(-)
		S. aureus, ATCC 25323	20 mg	(-)
		P. gingivalis, ATCC 33277	20 mg	27.0 (0.3)
tetracycline	2.1-2.3[a]	*E. coli*, ATCC 25922	30 μg	16.4 (0.3)
		S. aureus, ATCC 25323	30 μg	23.7 (0.3)
		P. gingivalis, ATCC 33277	30 μg	45.0 (2.9)
metronidazole	5.8[a]	*E. coli,* ATCC 25922	30 μg	(-)
		S. aureus, ATCC 25323	30 μg	(-)
		P. gingivalis, ATCC 33277	30 μg	75.0 (1.5)

Sample 1 (crude), lyophilization of spent culture broth; Sample 2 (semi-pure), lyophilization of spent culture broth after dialysis; Sample 3 (pure), lyophilization of fraction no. 71–82 after gel permeation; pH, pH of aqueous solution of Samples 1–3 or of antibiotics; R, resistant (inhibition zone ≤8 mm); –, no inhibition zone; ND, not determined.

[a] Values from the Merck Index.

Table 3-2 Antibacterial activity spectrum of bacteriocin produced by *L. paracasei* HL32 compared with metronidazole as a reference

Bacterial strains	Mean (SD) zone of inhibition (mm)					
	metronidazole	Bacteriocin treated with enzyme				
			enzyme			
		Without	Trypsin	Chymo trypsin	Lipase	Amyloglu cosidase
P. gingivalis ATCC 33277	48	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)	R	(-)	(-)	(-)	(-)
T. forsythensis ATCC 43037	70	(-)	(-)	(-)	(-)	(-)
S. salivarius ATCC 25975	(-)	(-)	14.1(0.2)	17.1 (0.2)	(-)	(-)
S. sanguinis ATCC 10556	(-)	(-)	(-)	(-)	(-)	(-)

-, no inhibition zone; R, resistant (inhibition zone 7.0-8.0 mm).

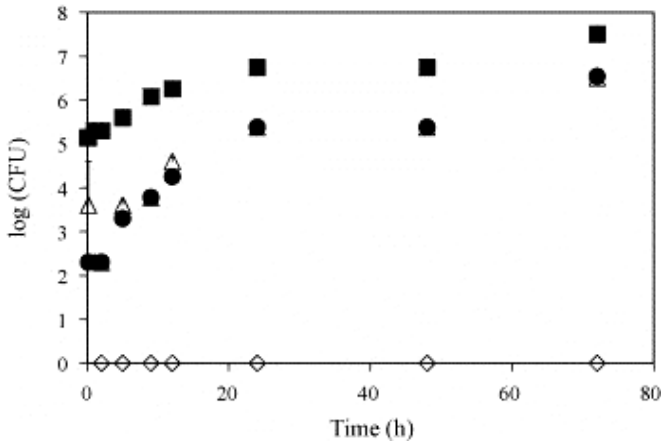


Figure 3-4 Kinetic death rate of bacteriocin produced from *L. paracasei* HL32 culture: *P. gingivalis* ATCC 33277 without the bacteriocin (■); *P. gingivalis* treated with pure bacteriocin 0.035 mM (△); *P. gingivalis* treated with pure bacteriocin 0.07 mM (●); *P. gingivalis* treated with pure bacteriocin 0.14 mM (◇). CFU, colony-forming unit.

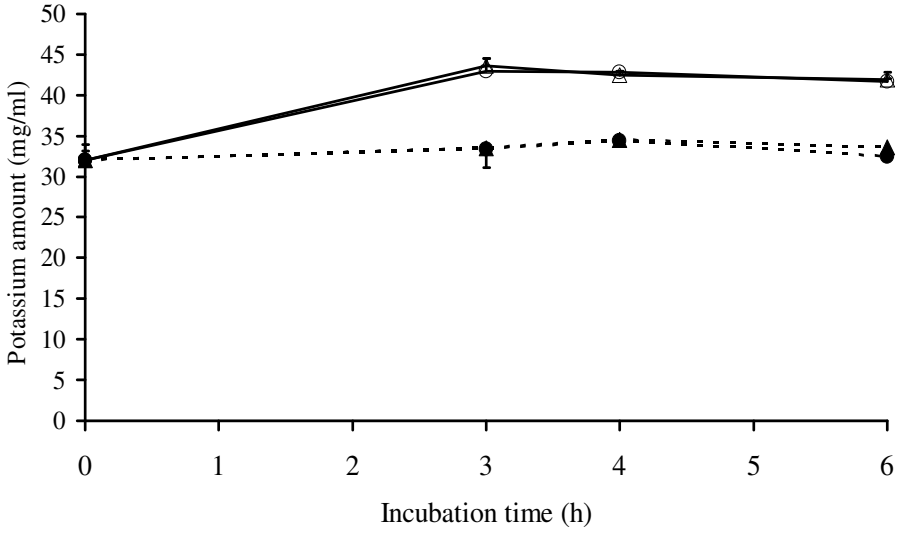


Figure 3-5 Potassium efflux from *P. gingivalis* ATCC 33277 and WP 50 induced by bacteriocin at MIC: (---) represent *P. gingivalis* ATCC 33277 (▲) and WP50 (●) without the bacteriocin ; (——) *P. gingivalis* ATCC 33277 (Δ) and WP 50 (○) treated with bacteriocin 0.14 mM.

Potassium leakage

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 at the level of the MIC (solid line). There were no differences in potassium levels between the supernatants, in the presence or absence of bacteriocin when incubation was at initial (Fig.3-5).

Scanning electron microscopy

SEM shows the effects of bacteriocin, tetracycline, metronidazole and lactic acid on the cell wall of *P. gingivalis* (Fig.3-6(A)). *P. gingivalis* untreated with bacteriocin exhibited coccoid to coccobacilli forms, with a diameter of about 0.3 μm. Bacteriocin (Sample 3, pure)-treated *P. gingivalis* were swollen into rod shapes, and pore formation was observed at the terminus of the rods (see Fig.3-6(B), inset). Tetracycline-treated *P. gingivalis* showed prominent cell elongation. Metronidazole-treated *P. gingivalis* and lactic acid-treated (0.3 M lactic acid, pH 4) *P. gingivalis* had normal cell shapes, similar to the untreated group.

Transmission electron microscopy

TEM showed that the addition of bacteriocin induced a noticable change in sensitive cells. Exponentially growing cells of *P. gingivalis* had a typical structure of Gram-negative bacteria, with a thin, periplasmic formation. The presence of membrane associated vesicles with their distinctive trilaminar appearance were apparent in the control images, as shown by the thin arrow

heads in Fig.3-7 (A) and 3-7 (B), but were absent in cells treated with bacteriocin. After 2 h of exposure to the bacteriocin, TEM of *P. gingivalis* showed areas where the outer and inner membranes appeared to be disrupted, as indicated by thick arrows in Figs.3-7 (C) and 3-7 (D), whereas the cell membranes of control group remained intact. TEM of *P. gingivalis* exposed to the bacteriocin and *P. gingivalis* control group showed without apparent loss of the underlying cell morphology after 2 h incubation. The cytoplasm in both groups is homogenous in appearance, with no evidence of clumping or filamenting of cytoplasmic constituents, and no phage particles were observed.

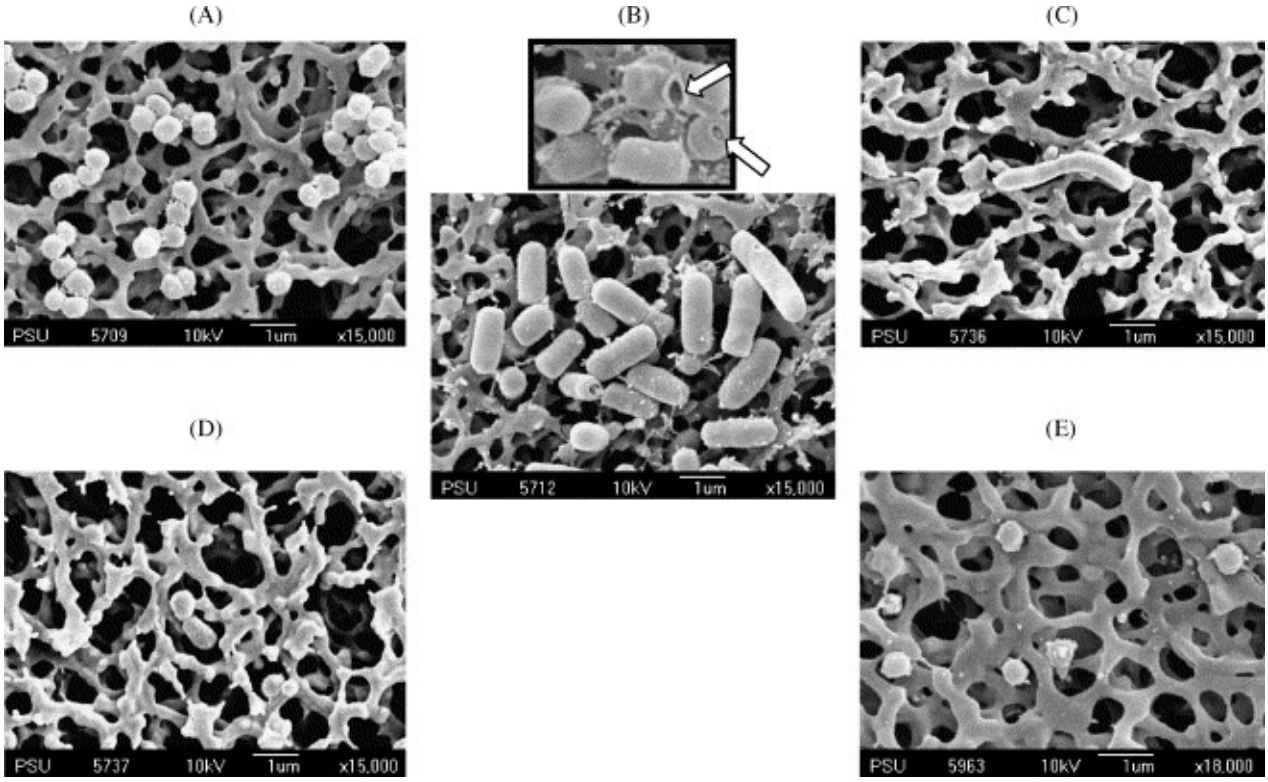


Figure 3-6 Cell wall deformations. SEM of *P. gingivalis* treated with each agent at bactericidal concentration: (A) untreated, (B) Sample 3 (pure) (0.14 mM), (C) tetracycline (1 mM), (D) metronidazole (0.14 mM), and (E) 0.3 M lactic acid, pH 4. An arrow indicates pore formation

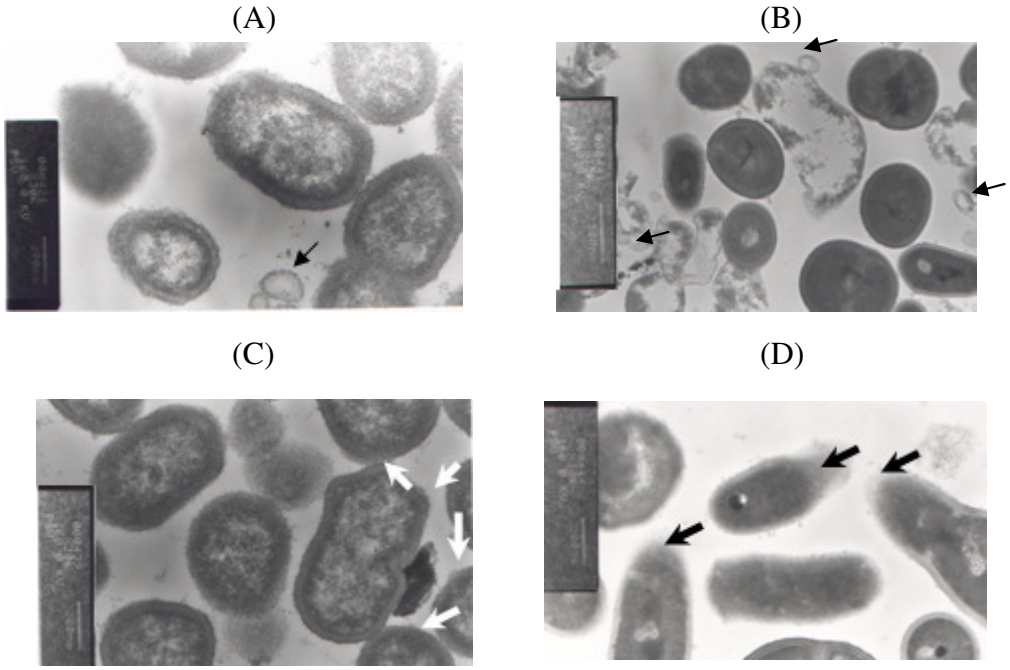


Figure 3-7 TEM of *P. gingivalis* (A) ATCC 33277 control (B) WP 50 control (C) ATCC 33277 + 0.14 mM bacteriocin (2 h) and (D) WP 50 + 0.14 mM bacteriocin (2 h). Bar = 200 nm in all figures

DISCUSSIONS

The sizes of inhibition zone in antibacterial screening of sample 1, sample 2, and sample 3 obtained from *L. paracasei* HL32 culture were difference, and changes of antibacterial spectra were seen (Table 3-1). The sizes of inhibition zone and broader antibacterial spectra of Sample 1 (crude) powder was in line with previous reports (Huttunen *et al.*, 1987; Kabuki *et al.*, 1997; Onda *et al.*, 2003). In the current study, the dialysed powder (Sample 2, semi-pure) had a narrower spectrum of activity than the crude powder, since the biosynthetic substance(s) of *L. paracasei* HL32 (Kaewnopparat, 1999) known to give a broader activity spectrum (lactic acid and

propionic acid) were removed during the purification steps (Schillinger and Lucke, 1989; Daeschel *et al*., 1990). The results also confirmed that antibacterial activity of the bacteriocin was not directly lactic acid and propionic acid, because Sample 3 (pure) powder completely lost its inhibitory activity against *E. coli* and *S. aureus*, and gave the largest inhibition zone (27.0 mm) on *P. gingivalis*. From this line, we concluded that the sample 3 (pure) powder has a narrow antibacterial spectrum against Gram-negative pathogen *P. gingivalis*. Even bacteriocins from Gram-positive bacteria including *Lactobacillus* rarely exert activity against Gram-negative bacteria.

Other periodontal pathogens and indigenous oral strains were included in the study to evaluate the specificity of the bacteriocin (Table 3-2), the selection of the indicator strains (Gram-positive bacteria: *S. sanguinis* and *S. salivarius*) was based on their importance in the oral ecosystem as members of the dominant indigenous normal flora (Fujimura and Nakamura, 1979; Hillman *et al*., 1985). *In vitro*, *S. sanguinis* and *S. uberis* have ability to produce hydrogen peroxide by converting oxygen, appear to promote periodontal health by keeping the numbers of potentially pathogenic organism (*P. gingivalis, P. intermedia, F. nucleatum, E. corrodens, Capnocytophaga sputigena*, and *A. actinomycetemcomitans*) below the threshold level necessary to initiate disease (Hillman *et al.*, 1985; Leke *et al.*, 1999). Unfortunately, the sample 3 (pure) gave the inhibition zone only both strains of *P. gingivalis*. These results revealed that the *L. paracasei* HL32 produced an antibacterial compound in which

the purified compound specifically inhibited only *P. gingivalis* strains. This bacteriocin seems to bear a great potential in combating dental pathogens.

The MICs obtained for tetracycline and metronidazole against *P. gingivalis* in this study, which were positive controls, were similar to those reported previously (Wade & Addy, 1987; Andres *et al.*, 1998). Sample 3 (pure) powder exhibited a time-dependent bactericidal effect in the present study. At 0.14 mM, it exhibited a bactericidal action against *P. gingivalis* within 2 h of incubation. Death rate kinetics of *P. gingivalis* were observed with 5×10^4 cells/h when incubated with MBC. Using lower concentrations (0.035–0.07 mM), *P. gingivalis* could not be killed completely; the remainder appeared to multiply in number after 2 h (Fig. 3-3).

Large number of bacterocins act by pore-forming (Abee *et al*., 1994; Gonzalez *et al*., 1994; Tahara and Kanatani, 1996; Duche, 2002; Castellano *et al*., 2003). SEM and TEM of *P. gingivalis* exposure to drugs gave important information regarding how the morphology and the internal structure of *P. gingivalis* was affected. In general, the mechanism of action of this antimicrobial protein may involve the formation of ion channels in the microbial membrane (Boman, 1991; Javadpour *et al.*, 1996), or competitive inhibition of adhesion of microbial proteins to Toll-like receptors (Zasloff, 2002). It was observed that the bacteriocin changed the *P. gingivalis* cell envelope from coccoid or coccobacilli forms to swollen rods with associated pore formation (Fig.3-4(B), inset). It is likely that growth (i.e. expansion of peptidoglycan) associated

with slower cell division as well as hypotonic condition led to the apparent swelling. Together with the pore formation that occurs, these two factors may combine to cause cell death. In contrast to tetracycline, metronidazole, and lactic acid, tetracycline is capable of inhibiting mucopeptide synthesis, which is part of the cell wall backbone, resulting in the cell wall elongation observed in *P. gingivalis* (Fig.3-4(C)). Metronidazole is able to kill organisms that inhabit a low redox environment, and thus are capable of anaerobic metabolism, by gaining entry into the micro-organisms and interrupting DNA synthesis, resulting in cell death (Edward, 2001). Therefore the morphology of metronidazole treated *P. gingivalis* is not affected. The lactic acid-treated *P. gingivalis* results demonstrate that lactic acid (0.3 M, pH 4) is able to kill the organism, but does not affect the morphology of the bacterial cell envelope.

The perforation of the cell membrane and the absence vesicle as viewed by TEM and SEM observation, 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 and therefore potassium leakage might be expected to occur as a secondary event (Abee, 1995; Montville *et al.*, 1995). The absence of vesicles as determined by TEM also confirmed that bacteriocin showed lysis activity against *P. gingivalis*'s membrane.

Interestingly, the antibacterial activity of bacteriocin was transformed after enzymatic treatment. The bacteriocin was inactived after incubation with trypsin and chymotrypsin, but after treatment with either enzyme it showed activity against *S. salivarius*. Normally, the trypsin activity cleaves amide and ester bonds of substrate specificity based upon

positively charge of arginine (Arg) and Lysine (Lys) side chains. The transformation of antibacterial activity can be concluded that Lys and Arg are important for the specific ligand binding conformation to either bacterial cell surface or bacterial receptor. Furthermore, the previous study confirmed that cationic characterization of Lys and Arg is valuable for antimicrobial activity of peptide (Ryadnov *et al.*, 2002; Javadpour *et al.*, 1996; Kondejewski *et al.*, 1996). In addition, the activity was reduced by the action of amyloglucosidase, indicating that the bacteriocin is likely to contain carbohydrate. Nevertheless, deglycosylated bacteriocin still retained bactericidal activity against both strains of *P. gingivalis*.

We suggested the first step in bacteriocin mode of action is bacteriocin adsorption. The difference in time between attachment of bacteriocin and microscopically detected cell lysis propably represented the time required for penetration of the bacteriocin into the cell envelope, where the lethal effects appeared to occur within the leakage of intracellular potassium which is the major intracellular cation in bacteria, then followed by cell expansion. Since K^+ acts as a cytoplasmic-signalling molecule, activating/or inducing enzymes and transport systems that allow the cell to adapt to elevated osmolarity (Bruno and Montville, 1993; Tahara and Kanatani, 1993) and examination of cells under light microscope showed that morphological changes could be seen within 48 h after exposure to the bacteriocin.

OBJECTIVE 3 to characterize and identify the bacteriocin obtained from *L. paracasei* HL32

RESULTS

TLC, SDS-PAGE and ESI-MS

TLC of Sample 1 (crude) powder revealed a mixture of proteinaceous substances, whereas both Sample 2 (semi-pure) and Sample 3 (pure) powders gave a single spot with R_f 0.6 corresponding to the bacteriocin (see Fig.3-1(B)). Including two chromatograms response to purified protein from AnIEx (anion exchange, non-binding) (R_f = 0.6 min), the first peak was the spotting area (Fig.3-8).

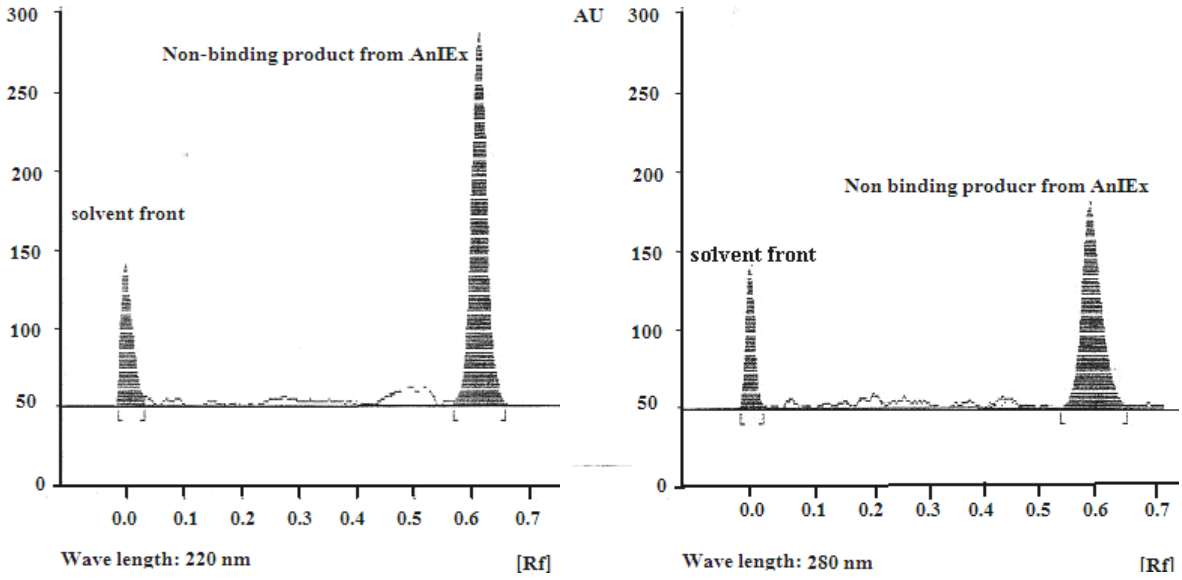


Figure 3-8 TLC chromatogram of non-binding product from AnIEx at 220 and 280 nm and TLC spot under 254 nm veiwer

SDS-PAGE electrophoresis of the Sample 1 (crude) showed two major bands with a MW between the range of 112-116 kDa and minor protein bands corresponding to smaller molecular weight (Fig. 3-1(D)). Further purification involving elution of the crude sample through a Superdex column produced the Sample 2 (semi-pure) where the two major bands were still detected but other species which produced minor bands corresponding to a MW of 16-45 kDa were also present. SDS-PAGE of Sample 3 (pure) stained with Coomassie blue G-250 showed a protein band (light blue color) appearing at an approximate molecular mass of 114 kDa (see Fig.3-1(C)), while ESI-MS mass spectrometry of the Sample 3 from preliminary small scale and fermentor scale gave a single sharp peak at 56 kDa and 57 kDa, respectively (Fig.3-9 (A) and (B)). In addition, the eluant product obtained after elution from an anion exchange column exhibited a single band by electrophoresis, corresponding to 56 kDa and the protein retained antimicrobial activity (Fig.3-1(D)).

Amino acid analysis

The bacteriocin contained approximately 32% amino acid residues (% w/w, estimated residues), it has a trace of methionine whilst cysteine/cystine are absent: Asp/Asn (2.7%, 13), Ser (1.55%, 10), Glu/Gln (4.54%, 20), Gly (5.43%, 53), His (1.0%, 4), Arg (2.37%, 9), Thr (1.3%, 7), Ala (2.19%, 17), Pro (4.08%, 24), Tyr (0.19%, 1), Val (1.46%, 8), Lys (2.09%, 9), Ile (1.26%, 6), Leu (1.29%, 6), Phe (0.55%, 2) and Met (0.36%, 2). The amino acid composition of bacteriocin contains a

high proportion of glycine and hydrophobic amino acids (mainly proline and alanine). Cationic and anionic residues were also detected.

Deglycosylation of the bacteriocin

Before amyloglucosidase digestion, the bacteriocin gave positive results to Seliwanoff's resorcinol test at 5 min, and gave a brownish precipitate with the Benedict's sugar test. After deglycosylation, it still gave similar results for Seliwanoff's resorcinol test, but the precipitate with the Benedict's sugar test was orange-red. This means that part of the bacteriocin is glycosylated. The molecular weight of the bacteriocin by ESI-MS after digestion was mainly at 17.5 kDa in Fig.3-9 (B). It also found small fragment of 12.6 and 16 kDa.

RP-HPLC

Fig.3-10 shows a chromatographic profile of the under this study. This figure also compares a chromatogram of the Sample 1 (crude), Sample 2 (semi-pure), Sample 3 (pure), and non-binding product obtained from AnIEx. Apparently, the interested compounds were eluted at retention time of 25 to 30 min. Furthermore, peak purity were performed using the photodiode array detector to demonstrate that the chromatographic peak of the non-binding product is pure.

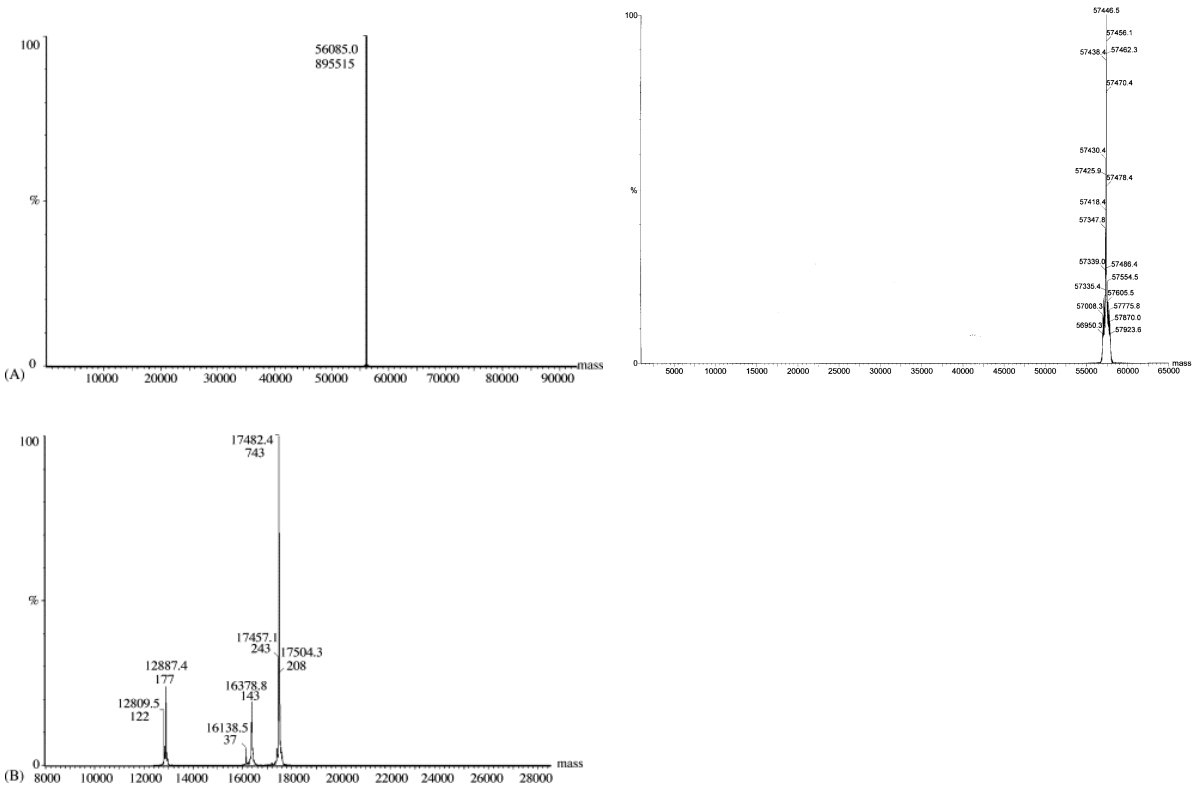


Figure 3-9 Mass spectrum of Sample 3 (pure) from recorded on ESI-MS: (A) before, (A_{left}: from preliminary scale; A_{right}: from fermentor scale) and (B) after deglycosylation.

Protein sequencing

- N-terminal sequencing

The full sequence of residues 1-156 of the bacteriocin as determined by automatic 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 identical to amino acid analysis information.

Later on, the primary sequence corresponding to bacteriocin was searched against the SWISSPORT and Protein Data Bank databases using both FASTA and BLAST. The bacteriocins show high sequence identity with Phage tail fiber-like protein from Cyanophage P-SSM2 (Q58MY1_9CAUD), Alpha collagen type 1 from *Paracentrotus lividus* (Common sea urchin) (Q26052_PARLI) ,or possible large adhesin [Haemophilus somnus 129PT], (ref|YP_719837.1|), or Bacteriophage Mu Transposase Core Domain (pdb|1BCO|), or The First Crystallographic Structure of a Xylanase from Glycosyl Hydrolase Family 5: Implications For Catalysis (pdb|1NOF|A), Chain A, Solution Structure Of The Nudix Enzyme Diadenosine Tetraphosphate Hydrolasef from *Lupinus angustifolius* L. (pdb|1F3Y|A) with quite low score of 41.346, 40.385, 35.4, 25.4, 24.6, 24.6 bits, respectively. However, this sequence did not show clear homologies with known bacteriocins.

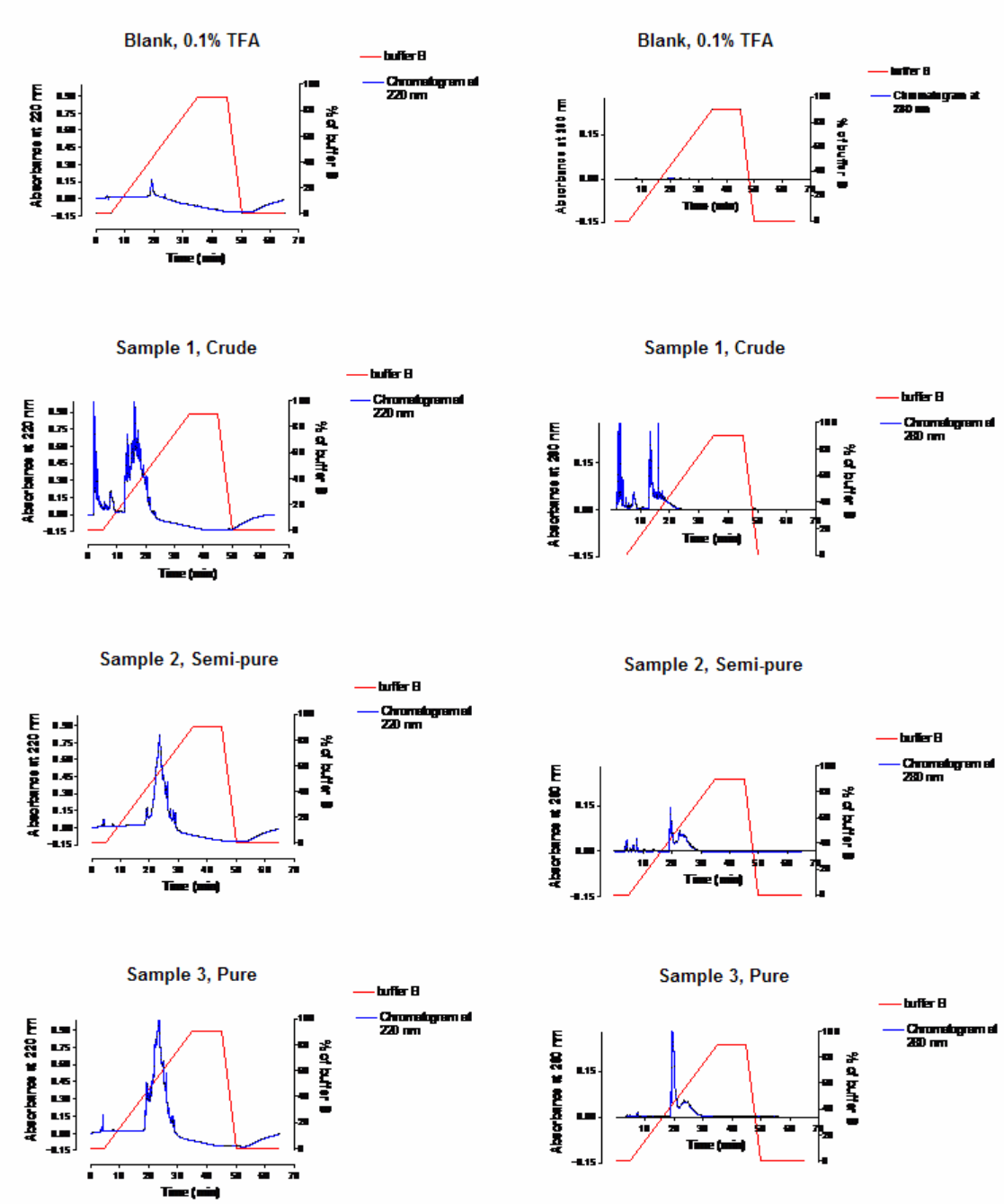


Figure 3-10 RP-HPLC Chromatogram of the blank (0.1% TFA), sample 1 (crude), sample 2 (semi-pure), sample 3 (pure) at 220 (left) and 280 nm (right)

- MALDI-TOF MS/MS

Mass spectrometry in the positive mode gave 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, 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, 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 (Fig. 3-11), respectively.

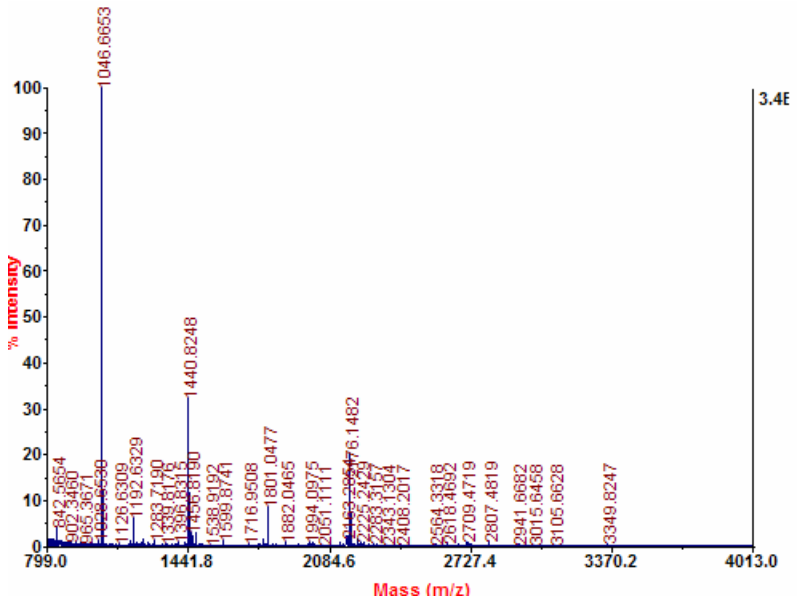


Figure 3-11 MALDI-TOF obtained from tryptic digestion of purified bacteriocin isolated from *L. paracasei* HL32

Then the mass spectrometries were compared to construction of a database of fragment masses used database from peptide mass fingerprints at www.matrixscience.com and prospector.uscf.edu/ ucsf.html3.4/msfit.htm as shown in Table 3-3.

Furthermore, the singly charged product ions, *m/z* 812.31, 1171.60, 1192.63, 1192.63, 1208.63, 1224.62, 1255.85, 1283.72, 1365.73, 1454.81, 1599.87, 2176.15, 2233.25, 2263.29, and 2825.49, were found to be corresponded to the calculated mass which are consistent with the bacteriocin primary sequence obtained by the Edman degradation as shown in Table 3-4.

Table 3-3 Matching peptide between fragment masses obtained from MALDI-TOF and database from peptide mass fingerprints

	Accession	Mass	Score	Description
1.	gi|6759482	17804	68	putative two-component response regulator [Bacillus cereus ATCC 14579]
2.	gi|116334903	51765	63	tRNA modification GTPase [Candidatus Carsonella ruddii PV]
3.	gi|29898943	15710	59	Two-component response regulator yocG [Bacillus cereus ATCC 14579]
4.	gi|115660786	54845	52	PREDICTED: similar to OTU domain containing 5 [Strongylocentrotus purpuratus]
5.	gi|124003622	8353	52	hypothetical protein M23134_03281 [Microscilla marina ATCC 23134]
6.	gi|40445356	178801	51	helicase [Gordonia westfalica]
7.	gi|47209843	39973	51	unnamed protein product [Tetraodon nigroviridis]
8.	gi|109460363	178240	51	PREDICTED: similar to Dynamin-binding protein (Scaffold protein Tuba) [Rattus norvegicus]
9.	gi|89091254	95557	50	DEAD/DEAH box helicase, N-terminal [Lactobacillus reuteri JCM 1112]
10.	gi|82534840	23507	50	hypothetical protein Bpse110_02003917 [Burkholderia pseudomallei 1106b]
11.	gi|76799484	92026	50	Sialidase A precursor (Neuraminidase A) [Streptococcus agalactiae 18RS21]
12.	gi|77414254	93070	49	neuraminidase-related protein [Streptococcus agalactiae 515]
13.	gi|22538070	93073	49	neuraminidase-related protein [Streptococcus agalactiae 2603V/R]
14.	gi|114707393	47948	49	DNA polymerase IV [Fulvimarina pelagi HTCC2506]
15.	gi|78046098	36437	49	transcriptional regulator, LysR family [Xanthomonas campestris pv. vesicatoria str. 85-10]
16.	gi|42741703	8522	48	thioredoxin [Alcaligenes faecalis]
17.	gi|91774474	157826	48	CobN/magnesium chelatase [Methylobacillus flagellatus KT]
18.	gi|71897003	53171	48	CD36 antigen [Gallus gallus]
19.	gi|84105068	53146	48	fatty acid translocase [Gallus gallus]
20.	gi|115466100	59784	48	Os06g0125300 [Oryza sativa (japonica cultivar-group)]

Table 3-4 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	2178.05
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
RPQGRDGPGQRPHGAGEGIDR	41 to 61	2233.25	2477.154
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

CD spectroscopy

CD spectrum of the Sample 3 has one negative absorption peaks at 200 nm (curve 1, Fig. 3-12).

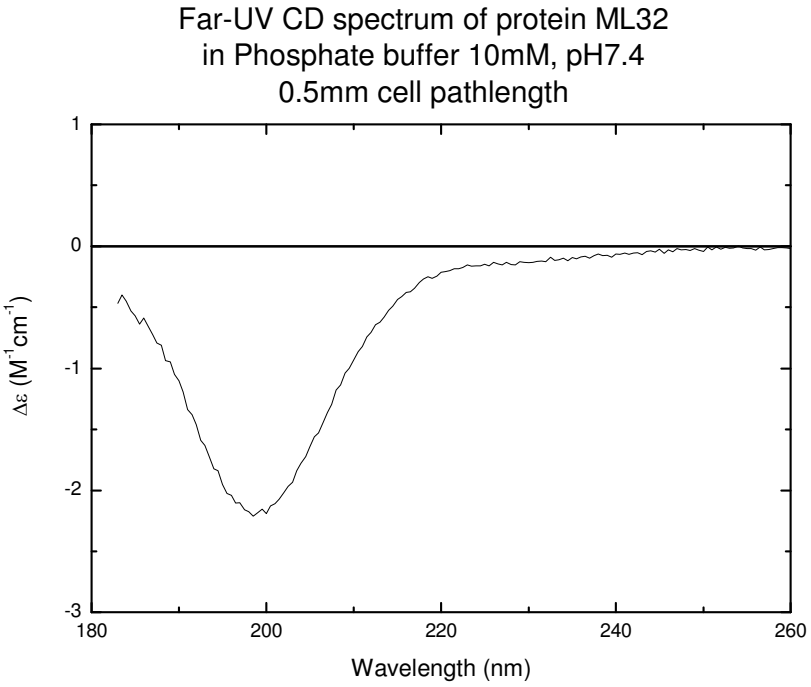


Figure 3-12 Random coil structure from CD analysis of bacteriocin

The secondary structure prediction of the bacteriocin using algorithms is presented in Fig.3-13. There are similar between the CD spectroscopy information and the prediction results, the bacteriocin appears to form random coil as a whole molecule.

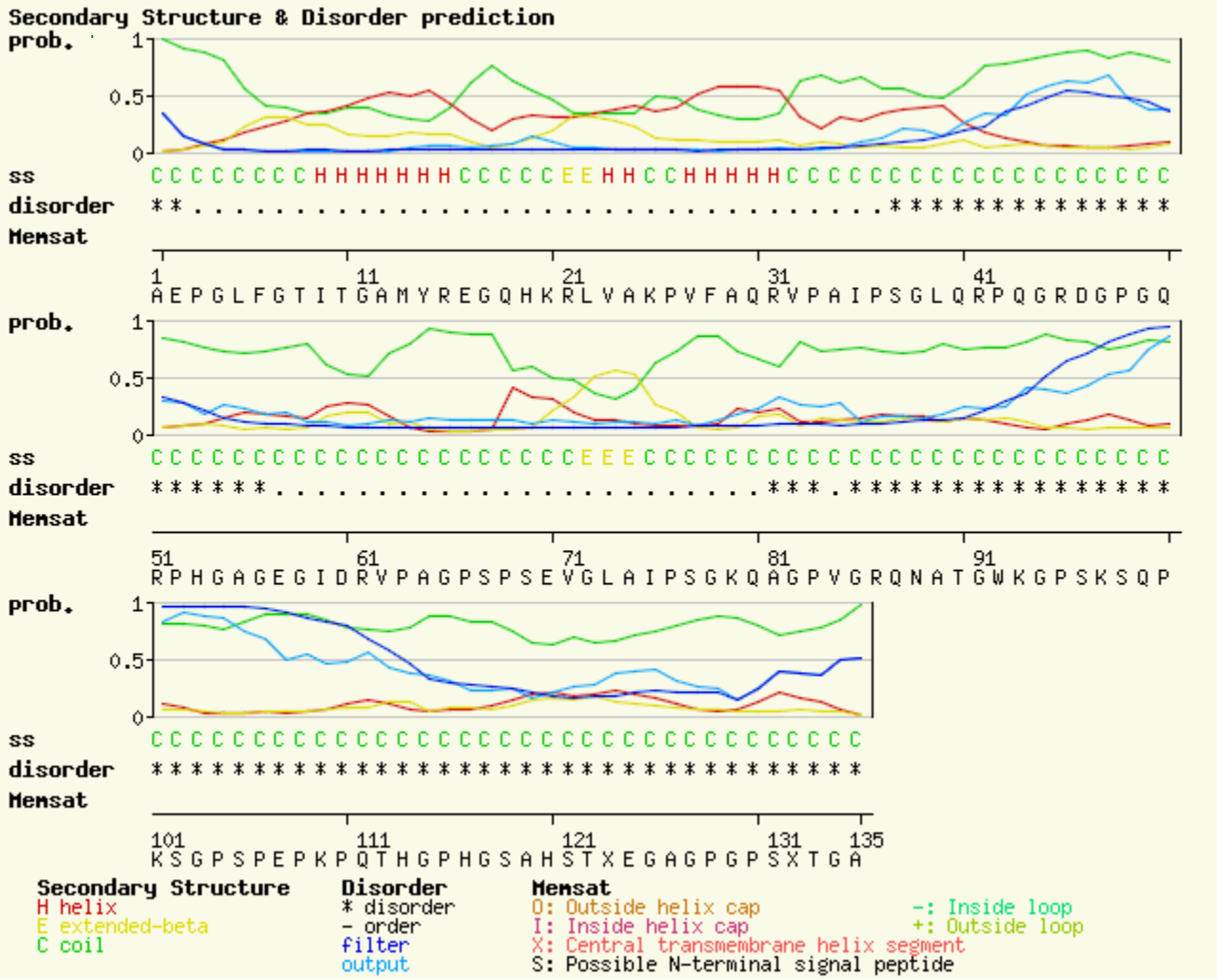


Figure 3-13 Secondary structure prediction of bacteriocin obtained from *L. paracasei* HL32

(Secondary structure prediction programs web address: http://swissmodel.expasy.org)

DISCUSSIONS

UV-absorption methods have the advantage of being rapid and nondestructive and are suitable for the bactericoin determination. As stated in chapter 1, absorbance determinations based on the wavelength of peptide bond (206-220 nm). In contrast to Lowry method, Folin-phenol reagent, and determination of A_{280} are not suitable for this bacteriocin which composes more than half of aliphatic amino acids and proline, sine these methods based on the presence of aromatic amino acids. However, the Lowry method is still used because of its simplicity, sensitivity, and precision. Protein dying binding method (Coomassie Brilliant Blue) react poorly with proline-rich protein (Walker, 1994; Lesk, 2004) and thus underestimates the concentration of total bacteriocin in gel after electrophoresis (SDS-PAGE) (Fig.3-1).

Although ESI-MS is more powerful than SDS-PAGE for molecular weight determination (Kabuki *et al.*, 1997), both of these techniques were utilised for data confirmation. The molecular weight as determined by SDS-PAGE was found to vary depending upon the purification procedure employed. The crude product migrated as a protein on SDS-PAGE with a MW of 114 kDa, however, a more purified product showed a single band corresponding to a MW of around 56 kDa. The latter MW was correlated well with that obtained with ESI-MS. The molecular mass of the bacteriocin was estimated to be 114 kDa by SDS-PAGE and 56 kDa by ESI-MS, suggesting that the bacteriocin is in a dimeric form (Fig.3-1(D)) or may be due to the fact that the antimicrobial compound associate with other cellular components under non-

dissociating conditions, since this has been reported to occur previously (Mortvedt *et al.*, 1991; Onda *et al.*, 2003).

Among the enzyme tested, the antibacterial activity was lost on treatment with both trypsin and chymotrypsin (Table 3-2), separately, indicating its proteinaceous nature. The composition of the bacteriocin has been explored in detail. Amino acid analysis revealed that the bacteriocin contained only 32% amino acid residues, with 18 kDa of calculated molecular mass of all the amino acid residues. On treatment with lipase, there was no reduction in the antibacterial activity. This observation ruled out the possible role of lipid composition in bringing out the inhibition of *P. gingivalis*. In an effort to examine the composition of the rest of the molecule, the bacteriocin was cleaved by amyloglucosidase (from *Aspergillus niger*) and subsequently investigated by sugar tests. The polysaccharide parts of the bacteriocin were completely broken down to yield monosaccharides with positive results in 5 min for the Seliwanoff's resorcinol test. The presence of glucose was indicated by the Benedict's sugar tests. A molecular weight analysis of the major protein part after deglycosylation revealed the mass to be 17.5 kDa. Although products with masses of 12.8 and 16.3 kDa were also obtained, these minor products may be due to further hydrolysis of the 17.5-kDa product. 17.5 kDa is close to the calculated molecular weight (18 kDa).

Interestingly, no significant similarity to any amino acid sequence was observed from the NCBI database, whereas the antibacterial activity still remained after deglycosylation,

indicating that the the substance isolated from *L. paracasei* HL32, oriented by proteins and carbohydrates was a novel bacteriocin.

In comparison to bacteriocin obtained from *L. paracasei* subsp. *paracasei* strain M3 (Atanassova *et al.*, 2003), the amino acid composition is distinguishing difference from our bacteriocin. Considering in the detail of amino acid composition, the antimicrobial activity of the bacteriocin may be due to its unique composition, e.g. Asp and Glu as anionic residues providing a negative charge, and Arg and Lys as cationic residues providing a positive charge (Javadpour *et al.*, 1996; Kondejewski *et al.*, 1996; Ryadnov *et al.*, 2002). Our bacteriocin has Pro and Gly as backbone, cationic charge at N-terminus and anionic charge at C-terminus. The initial adherence properties of the bacteriocin on *P. gingivalis* may be due to electrostatic interaction between the cationic residues and acidic phospholipids on bacterial cytoplasmic membrane (Zasloff, 2002). Gly, Ala, Pro, Val, Ile and Leu confirm the hydrophobic character of the active bacteriocin, and probably facilitate a membrane insertion, then perform hydrophobic interaction between the hydrophobic residues and zwitterionic phospholipids on the cytoplasmic membrane, and thus formation of stable pores (Javadpour *et al.*, 1996; Kondejewski *et al.*, 1996; Zasloff, 2002). It is very interesting that Pro and Gly residues are found at regular intervals along a central part of the bacteriocin. This feature is able to form an amphiphilic structure, where the amphiphilic ability obtained from these amino acid residues has been found to be crucial for antibacterial activity (Javadpour *et al.*, 1996; Kondejewski *et al.*, 1996; Ryadnov *et al.*, 2002; Zasloff, 2002). In fact, Met may establish structure stability resulting in disulphide bond formation. Furthermore, Ala and

Leu have a high helical propensity (Javadpour *et al.*, 1996; Ryadnov *et al.*, 2002). The glycogenic part may provide ligand-binding receptors and stability to the bacteriocin structure.

Identification of the protein by proteolytic digestion with trypsin, for most proteins, the masses of their tryptic peptides form a set of numbers which is unique (or very nearly so). With such information existing databases, can be searched to identify the protein being studied. The protein found by matches their theoretical tryptic peptide masses with MS peak masses are rank-order according to the closeness of match using various algorithms. However, using protein database could not predict primary sequence of our bacteriocin. It possibly due to our bacteriocin is a novel protein.

All amino acid residues of bacteriocin were detected by N-terminal amino acid sequencing. The sequencing was not blocked, probably because the absence of modified amino acids residues such as lanthionine was contained in the structure, as described before (Mortvedt *et al*., 1991; Muriana and Klaenhammer, 1991; Tahara and Kanatani, 1996; Remiger *et al*., 1999). Protein structure prediction was success by N-terminal Edman degradation analysis and was confirmed by information obtained from combination of sequence analysis of tryptic fragment with mass spectrometry (Table 3-4).

Comparison of the amino acid sequence of the bacteriocin with those of other bacrteriocins in FASTA and BLAST revealed very weak homology to the partial sequence

obtained from each resources with highest score only 41.346. The non homology between bacteriocins produced from *L. paracasei* HL32 confirmed that they are novel protein as well.

Amino acid sequence shows the presence of cationic amino acids near the N-terminus which is quite similar to plantaricin C (Gonzalez *et al*., 1994), plantaricin 1.25β (Remiger *et al*., 1999), and a high percentage of hydrophobic amino acids in the central region (Leu, Ile, Val, Phe, Trp, and Gly). It also contains a highly charged C-terminus (Ser, Asn, and Gln). In former results from chapter 2, bacteriocin isolated from *L. paracasei* HL32 shows a limited host range. Taken together, it is suggested that the bacteriocin exert its action through interaction with cell wall-associated or membrane-associated binding sites in the sensitive cells. Our results suggest that its net positive charge at the 30 N-terminal amino acids (5+ at pH values lower than 6) would neutralize the negative charge of teichoic and lipoteichoic, function as initial binding at the outer membrane of *P. gingivalis* cells and may be needed for an electrostatic interaction, and interact specifically. The outer membrane does control the transport of certain proteins from the environment and its outer surface has antigen and receptor (Tortora *et al*., 1998; Holt *et al*., 1999). Probably certain bacteriocin may bind to some receptors as the initial binding and then fold to act via specific receptor or docking site (Power and Hancock, 2003). For that reason, the bacteriocin may interact competitively with some target cell entity, which would then be followed by membrane insertion of the uncharged portion of C-terminal part the bacteriocin into the hydrophobic part of target-cell membranes like other reported bacteriocin (Fimland *et al.*, 1996; Kawai *et al*., 2001) allowing the bacteriocin to interrupt the cell wall synthesis at the log

phase of *P. gingivalis*, which increase membrane permeabilization and cause cell deformation and then death, as shown in Fig.3-14.

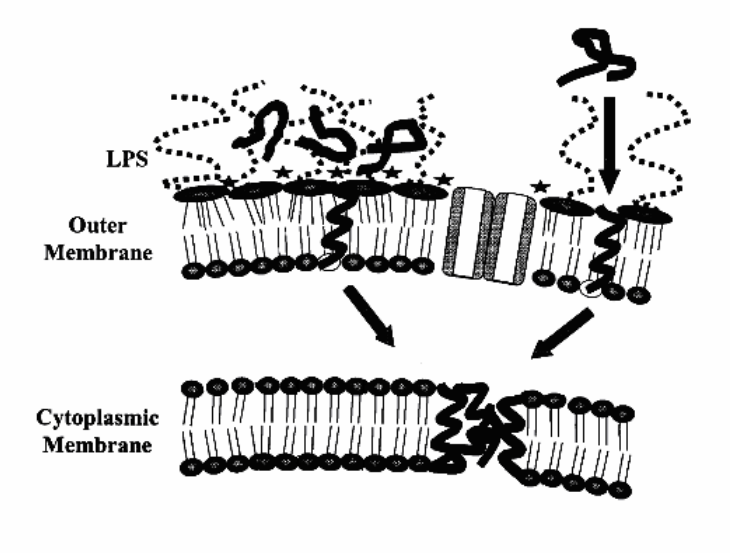


Figure 3-14 Proposed mechanism of interaction of cationic antimicrobial peptides with the cell envelope of Gram-negative bacteria
(Modified from Powers, J.P., and Hancock, R.E.W. 2003. The relationship between peptide structure and antibacterial activity, Peptides, 24**:** 1681-1691.)

The absorbance of peptide bonds is usually located in the far ultraviolet range (Copeland, 1994; Walker, 1994). Therefore, the absorption peak in the far ultraviolet range can reflect the conformation changes of peptide chains of protein. Since in the sequencing has no thiol containing amino acids, it may be not induce secondary structure. In addition, the sequence of our bacteriocin has a lot of proline/glycines/serines, there is unlikely to be any secondary structures and though to confer a high degree of conformational freedom. The analysis of soluble bacteriocin by CD shows a clear minimum for the spectrum of the soluble sample at 200 nm, indicating a mostly random coil structure.

It has been postulated that bacteriocins with fewer amino acid residues and the presence of an extra disulfide bond would tend to have a relatively broader antibacterial spectrum (Ennahar *et al.*, 1999). In similarity, the bacteriocin in this study composes of several amino acid residues (156 residues) without disulfide bond has the limited antibacterial activity.

OBJECTIVE 4 to evaluate the effects of pH, temperature, and biological stability on bacteriocin activity

RESULTS

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

The bacteriocin remained soluble and active over a wide range of pH 2.6-9.0. A reduction in bactericidal activity was observed following heat treatment, but even autoclaving the bacteriocin in solution at 121 ^{o}C for 15 min reduced activity by only 4-8%. There was no statistically significant difference the % inhibition obtained after heat sterilization at 100 ^{o}C for 60 min and that obtained following filter sterilization (*P=0.841*) (Table 3-5). However, the stability of paracasin HL32 to heat treatment showed the inhibitory activity was not altered after 60 min at 50 ^{o}C, 80 ^{o}C, or 100 ^{o}C. There was also no significant reduction in activity upon storage of bacteriocin at 4 ^{o}C for 6 month (data not shown). In contrast, at 25 ^{o}C (room temperature) and at 37 ^{o}C, the bacteriocin activity was found to be lost after 11 weeks. Antibacterial activity of the bacteriocin was destroyed by incubation with trypsin and chymotrypsin, but there was little reduction in activity as a consequence of incubation with either amyloglucosidase or lipase (Table 3-2).

Table 3-5 Hemolytic potential of bacteriocin on human erythrocytes following a 24 h exposure time and the effect of heat, pH, and biological fluid on the activity of bacteriocin

Treatment	% inhibition (±SD)	*% hemolysis* (±SD)	*P-value*
Bacteriocin 0.14 mM	83.40 (0.45)	0 (0.0)	
0.28 mM		16 (0.3)	
0.56 mM		100 (0.0)	
Melittin 0.625 μM		100 (0.0)	
pH:			
2.2-6.8 (acidic condition)	83.19 (3.64)		
7.4 (physiological pH)	84.13 (0.78)		
8.5-9.0 (basic condition)	83.31 (3.73)		
Heat:			
100°C for 60 min	83.57 (2.18)		0.841
110°C for 30 min	80.10 (0.43)		0.007
121°C for 10 min	79.89 (1.96)		0.007
121°C for 12 min	75.78 (4.72)		0.015
121°C for 15 min	75.15 (3.56)		0.015
Biological fluid			
A, Saliva (healthy volunteers)	66.86 (7.11)		A and C, 0.0005
B, Saliva (periodontitis patients)	71.71 (9.85)		B and A, 0.1484
C, GCF (healthy volunteers)	72.46 (5.85)		C and D, 0.4609
D, GCF (periodontitis patients)	74.75 (6.38)		D and B, 0.3828

Porphyromonas gingivalis ATCC 33277 was used as indicator organism.

Biological activity

The antimicrobial activity of bacteriocin on *P. gingivalis* ATCC 33277 was decreased by 17% and 12% respectively when the microorganisms were incubated in saliva derived from healthy volunteers and periodontal patients, compared to controls. However this difference from was not significant. The % inhibition determined in the presence of gingival crevicular fluids obtained from healthy volunteers and periodontal patients was 11% and 9% less respectively compared to positive controls. Again there was no significance difference between these mean reductions, however, there was a statistically significant difference between the inhibition in the presence of saliva and gingival crevicular fluid obtained from the healthy volunteers *($P < 0.01$)* (Table 3-5).

DISCUSSIONS

Whole saliva consists of a mixture of secretions from the parotid, submandibular, sublingual, minor glands, plus GCF components, shed microorganisms and shed epithelial cells. In whole saliva, many important proteins (glycoproteins, agglutinating factor, blood group substances, statherin and proline-rich proteins, histidine-rich polypeptides and lactoferrin, immunoglobulins, and biologically active peptides), enzymes, low molecular weight components (electrolytes, glucose, ammonia and urea), lipids are found (Söderling, 1989). The number of enzymes (i.e. amylase, lipase, and proteases, see in Table 3-6) in whole saliva originates from oral microorganisms, other sources are the crevicular fluid, polymorphonuclear leukocytes, epithelial

cells, and dietary constitutes; however the α-amylase concentration in whole saliva is high and can be stored at -20°C (Table 3-6) (Söderling, 1989; Zelles *et al*., 1995). They are generally resistant to long-term storage in -20°C (Söderling, 1989).

Table 3-6 Pretreatment and storage of whole saliva for enzyme analyses

Enzyme	Pretreatment of saliva	Storage condition			
		+4°C		Frozen (-20°C)	
		Short-term[a]	Long-term[b]	Short-term[a]	Long-term[b]
α-amylase	Untreated or clarified	+[c]	-[d]	+	+
Lysozyme	untreated	-	-	+	-
	acidified	+	+	+	+
Peroxidase	Untreated or clarified[e]	+	-	+	+
Glycosidases	Untreated or clarified[e]	+	-	+	-
Esterase	Untreated or clarified[e]	+	-	+	-
Proteases and peptidases	Untreated or clarified[e]	+	-	+	+/-

Note: for further details and references, see text.

[a] short-term: a few days

[b] long-term: several weeks or months

[c] plus (+): the enzyme is generally stable

[d] minus (-): the enzyme may be unstable

[e] clarification decreases enzyme activity

GCF composes of proteins of serum origin, polymorphonuclear leukocytes and their products including albumin, transferring haemopexin and haptoglobins, tissue breakdown products, and subgingival bacteria and their products. Other growth factors are α_2-globulin, progesterone and oestradiol. Part of the diversity of the flora of the gingival crevice can be

attributed to the development of food chains whereby the products of metabolism of one cell become the primary nutrients of another. The components of GCF were summarized as below (Marsh, 1990; Chapple, 1997).

Bacterial products: lipopolysaccharide (endotoxins), urea, exo- and endo-peptidases(i.e. trypsin-like enzymes, collagenases, antigens/mitogens), and alkaline phosphatase

Host cell products: cytokeratins, arachidonic acid metabolites (i.e. prostaglandin, leukotrienes), iron (lactoferrin, transferrin, hemoglobin)

Host cell derived enzymes: serum derived enzymes (plasmin, plasminogen and products of the clotting, fibrinolytic systems, kallikrein and kinnins, complement components), cytoplasmic enzymes (aspartate aminotransferase, lactate dehydrogenase), lysosomal enzymes of the host cells (collagenase, gelatinase, elastases, cathepsins B, D and L, β-glucuronidase, arylsulphatase, lysozyme and lactoferrin, myeloperoxidase, protease inhibitors, tissue inhibitors of matrix metalloproteinases, acid phosphatase, alkaline phophatase)

Indicator of humoral/cell mediated immunity: IgG 1-4, IL 1-β, IL 6, TNF-α

Products of host connective tissue breakdown: collagen broken products (hydroxyproline), proteo/glycosaminoglycans (hyaluronan and chondroitin-4-sulphate), factors related to fibroblast activity, factors related to osteoblast/clast activity

In this investigation, the antimicrobial activity of bacteriocin was affected by saliva rather than gingival crevicular fluids. For that reason, the bacteriocin conformation may be

degraded at glycosidic bonds by amylase obtained from saliva. Probably proline-rich protein and proline-rich glycoprotein obtained from the secretion can be affected bacteriocin interaction by binding competition on the *P. gingivalis* surface, or blockage of the binding sites of the proline-rich bacteriocin (Atrih *et al.*, 2001). Qualitative and quantitative investigations on components in saliva and GCF obtained from volunteers have greatly contributed to a better understanding of the natural effects and significant results, but we did not identify yet. Measurement the amount of gingival fluid collected on paper strip by Periotron, an electronic machine for measuring gingival fluid absorbed on paper, may be useful and precise instrument for the future study (Griffiths *et al.*, 1988; Johansson *et al.*, 1994).

Bacteriocin activity may be reduced by pH. pH values from 2.6 to 5.6 had ability to inhibit the growth of *P. gingivalis* without any treatment (Takahashi *et al.*, 1997; Kaewsrichan *et al.*, 2004). However, this study discovered that when the concentration of buffer was reduced from 50 to 5 mM, the acidic pH values were not affected to the growth of *P. gingivalis*. Over wide range of pH, using the lower buffer concentration mimic to oral fluids (Bardow *et al*., 2000), there was no change in antimicrobial activity of bacteriocin. Even the N-terminal hydrophilic and cationic residues seem to be necessary for initial electrostatic interaction between the bacteriocin and the negatively charged cell membrane (Abee, 1995; Hancock, 2001; Zasloff, 2002); however this observation has found that the bacteriocin retained the antimicrobial activity in alkaline conditions. This result suggests that the electrostatic activity interaction may not be necessary for the initial binding for the bacteriocin, and bacteiroicn is practical to use as

topical application in the subgingival pocket where *P. gingivalis* strains usually grow at neutral pH but where the pH tends to rise to alkaline conditions during growth (Takahashi *et al.*, 1997).

For storage purpose, bacteriocin obtained from the temperature isolates remained stable after storage for 6 month at 4 ^{o}C, but declined or became non-detectable after storage for 11 weeks at room temperature and 37 ^{o}C, indicating that cold temperature may be the most appropriate preservation technique (McGinn, 1996).

The bacteriocin in this study appeared to be heat-stable, since it was able to withstand the effects of a high temperature over 100^{o}C for 1 h, with residual activity remaining (83.57%). Such heat stability is unusual, but is probably a result of protein glycosylation which generally provides resistance against degradation by heat treatment. Such heat stability would be advantageous for possible commercial development of the bacteriocin since sterilization by autoclaving could be contemplated, rather than more expensive filtration process. In addition, the bacteriocin resistance to autoclaving indicated that the antibacterial inhibitory effects are not a consequence of bacteriophage contaminant.

Even the bacteriocin isolated from *L. paracasei* HL32 has potential for being developed as narrow-spectrum antibiotics. However, bacteriocin might be limited by its sensitivity to proteases and amylases. In this study, the bacteriocin contact to the biological fluids composing of proteinases, complement, and immunoglobulin (Söderling, 1989; Johansson *et al.*, 1994), the bacteriocin retained the antibacterial activity. Therefore, the bacteriocin isolated from *L. paracasei* HL32 is a promising agent used in periodontal therapy.

OBJECTIVE 5 to evaluate cytotoxicity of the purified bacterocin

RESULTS

Hemolysis assay

Bacteriocin was found to be non-hemolytic at the MIC under the conditions employed in this study (Table 3-6). When erythrocytes were treated at two times of MIC, 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 24 h incubation. In comparison to melittin (venom protein), at 5 μM concentration caused complete hemolysis after incubation for only 1 h. The results were also observed under microscope and no deformation was observed.

Mammalian cell assays

No effect on the morphology and viability of fibroblast cells and periodontal ligament cells were observed for bacteriocin treatment up to 0.14 mM for 12 h. No significant decrease in viable cells was observed after 48 h for PDL cells and 24 h for fibroblast cells (Fig.3-15 and 3-16). Significant decrease in %viable cells at higher concentration was also observed. The toxic level of mellitin is obviously much lower than bacteriocin.

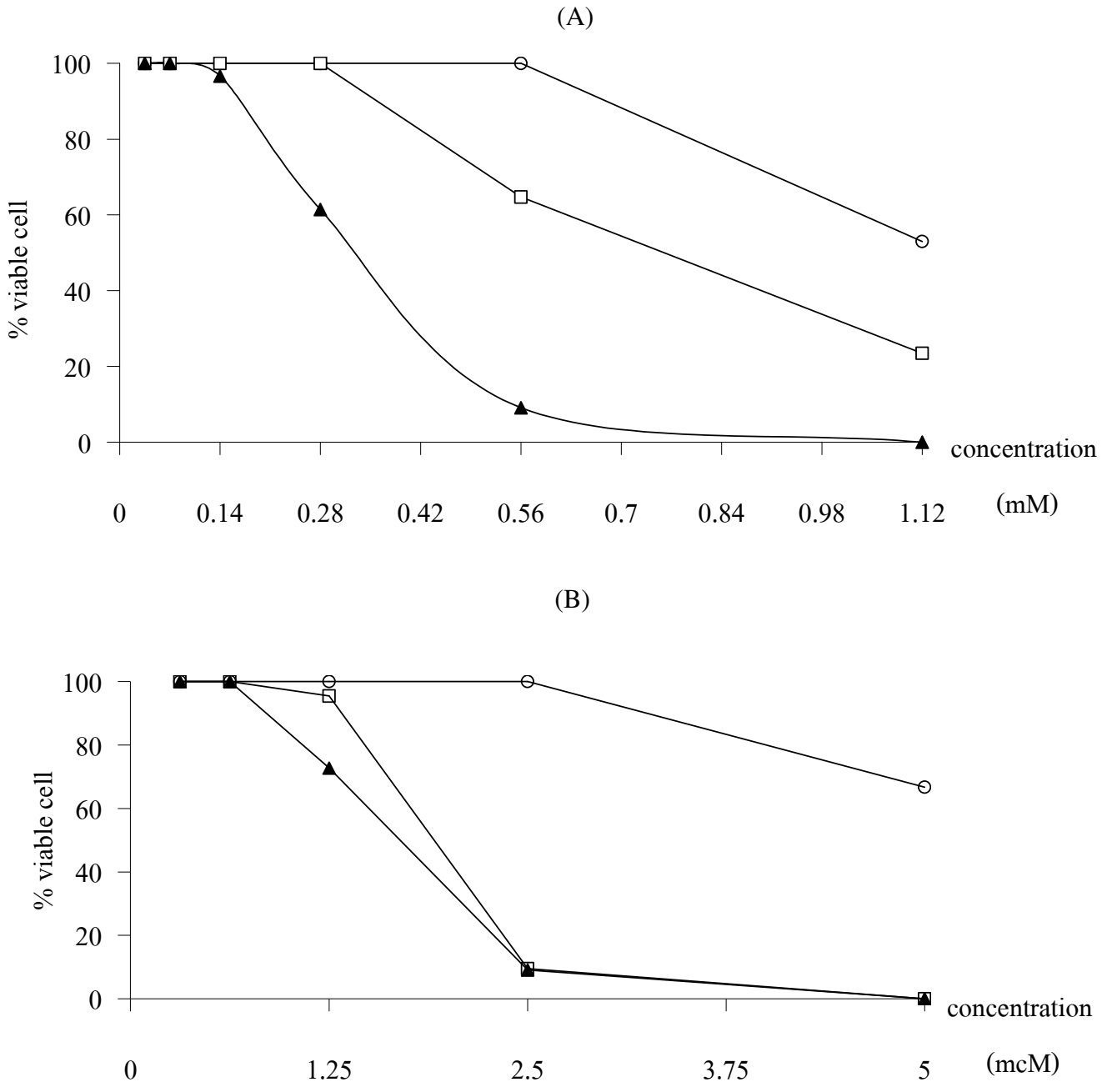


Figure 3-15 The effect of bacteriocin (upper) and mellitin (lower) on the viability of human periodontal ligament cells (n=3) after incubation for 2 h (○), 6 h (□), and 24 h (▲)

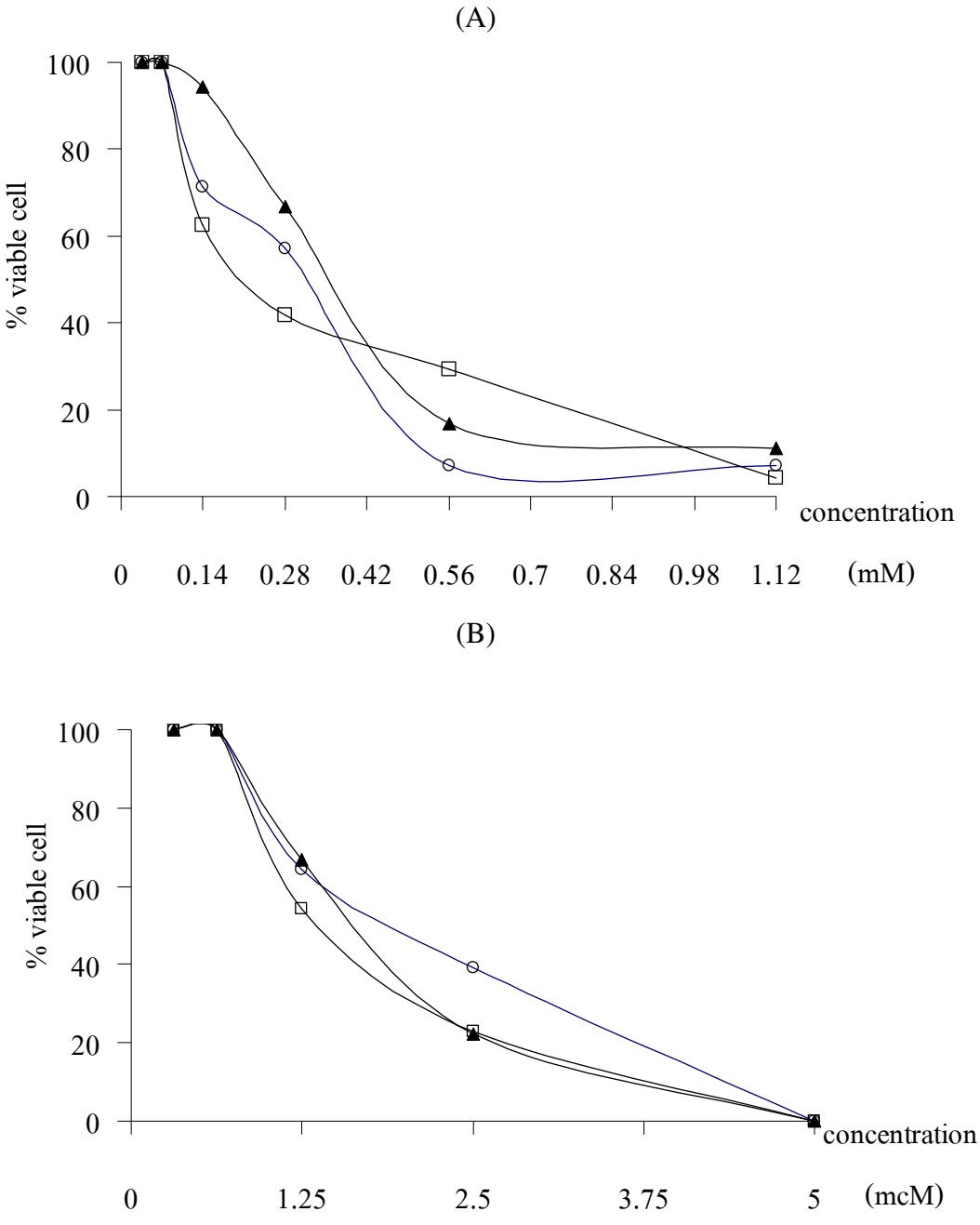


Figure 3-16 The effect of bacteriocin (upper) and mellitin (lower) on the viability of fibroblasts from gingival tissue (n=3) after incubation for 2 h (○), 6 h (□), and 24 h (▲)

DISCUSSIONS

The investigation of the *in vitro* cytotoxicity of the bacteriocin is essential before they can be considered in clinical use. Because erythrocyte plasma membrane is a natural membrane in the body which contains anionic surface charge and a lipid monolayer (Zasloff *et al*., 2002), it is good representation of prokaryoctye negatively charged cell containing lipid bilayer. In this study, the toxicity of bacteriocin was compared with melittin, a venom peptide derived from the bee. The bacteriocin was shown to induce hemolysis, but at concentrations much higher than the MIC, and was lesser cytotoxic and hemolytic than mellitin. The cytotoxicity was concentration- and time-dependent. More specifically, at low peptide concentrations (0.35, 0.7, and 0.14 mM), cell survival was independent on time. However, different sensitivity between the periodontal cells and fibroblast cells to bacteriocin was observed at 72 h when the concentration of the peptide was above 0.14 mM. The bacteriocion caused toxic at concentrations which 4-fold higher than those required for antimicrobvial activity, mellitin was toxic at concentrations in the same order of magnitude as its antimicrobial activity (Javadpour *et al*., 1996). Therefore, the bacteriocin has potential for use in antibiotics for the treatment of *P. gingivalis* infections.

Inspectation the pattern of the effect of bacteriocin and the mellitin on the viability of the human cells seem to be the same. We suggest the toxicity of the bacteriocin probably caused by hydrophobicity similar to mellitin one (Zasloff, 2002).

References

Andres,M., Chung,W., Roberts,M. & Fierro,J. (1998) Antimicrobial susceptibilities of *Porphyromonas gingivalis, Prevotella intermedia*, and *Prevotella nigrescens* spp. isolated in Spain. *Antimicrob Agents Chemother*, **42,** 3022-3023.

Boman,H. (1991) Antibacterial peptides: key components needed in immunity. *Cell*, **65,** 205-207.

Ennahar,S., Sonomoto,K. & Ishizaki,A. (1999) Class IIa bacteriocins from lactic acid bacteria: Antibacterial activity and food preservation. *Journal of Bioscience and Bioengineering*, **87,** 705-716.

Hillman,J.D., Socransky,S.S. & Shivers,M. (1985) The relationships between streptococcal species and periodontopathic bacteria in human dental plaque. *Archives of Oral Biology*, **30,** 791-795.

Huttunen,E., Noro,K. & Yang,Z. (1987) Purification and identification of antimicrobial substances produced by two *Lactobacillus casei* strains. *Int Diary J*, **5,** 503-513.

Javadpour,M.M., Juban,M.M., Lo,W.C.J., Bishop,S.M., Alberty,J.B., Cowell,S.M., Becker,C.L. & McLaughlin,M.L. (1996) De Novo Antimicrobial Peptides with Low Mammalian Cell Toxicity. *Journal of Medicinal Chemistry*, **39,** 3107-3113.

Kabuki,T., Saito,T., Kawai,Y., Uemura,J. & Itoh,T. (1997) Production, purification and characterization of reutericin 6, a bacteriocin with lytic activity produced by *Lactobacillus reuteri* LA6. *Int J Food Microbiol*, **34,** 145-156.

Kaewnopparat,S. Human lactobacilli as antidiarrheal and anticholesterol bio-agents: *in vitro* and *in vivo* studies. 1999. Faculty of Graduate Studies, Mahidol University, Bangkok.
Ref Type: Thesis/Dissertation

Kaewsrichan,J., Douglas,C.W.I., Nissen-Meyer,J., Fimland,G. & Teanpaisan,R. (2004) Characterization of a bacteriocin produced by Prevotella nigrescens ATCC 25261. *Letters in Applied Microbiology*, **39,** 451-458.

Kondejewski,L., Farmer,S., Wishart,D., Kay,C., Hancock,R. & Hodges,R. (1996) Modulation of structure and antibacterial and hemolytic activity by ring size in cyclic gramicidin S analogs. *J Biol Chem*, **271,** 25261-25268.

Leke,N., Grenier,D., Goldner,M. & Mayrand,D. (1999) Effects of hydrogen peroxide on growth and selected properties of Porphyromonas gingivalis. *FEMS Microbiology Letters*, **174,** 347-353.

Mortvedt,C., Nissen-Meyer,J., Sletten,K. & Nes,I. (1991) Purification and amino acid sequence of lactocin S, a bacteriocin produced by *Lactobacillus sake* L45. *Appl Environ Microbiol*, **57,** 1829-1834.

Onda,T., Yanagida,F., Tsuji,M., Shinohara,T. & Yokotsuka,K. (2003) Production and purification of a bacteriocin peptide produced by Lactococcus sp. strain GM005, isolated from Miso-paste. *International Journal of Food Microbiology*, **87,** 153-159.

Ryadnov,M., Degtyareva,O., Kashparov,I. & Mitin,Y. (2002) A new synthetic all-D-peptide with high bacterial and low mammalian cytotoxicity. *Peptides*, **2,** 1869-1871.

Takahashi,N., Saito,T., Schachtele,C. & Yamada,T. (1997) Acid tolerance and acid-neutralizing activity of *Porphyromonas gingivalis*, *Prevotella intermedia* and *Fusobacterium nucleatum*. *Oral Microbiol Immunol*, **12,** 323-328.

Wade,W. & Addy,M. (1987) Comparison of in vitro activity of niridazole, metronidazole and tetracycline against subgingival bacteria in chronic periodontitis. *J Appl Bacteriol*, **63,** 455-457.

Zasloff,M. (2002) Antimicrobial peptides of multicellular organisms. *Nature*, **415,** 389-395.