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Contributed Paper

Structural Characterization and Mode of Action Studies on Salvicin K and Antimicrobial Peptide-like Bacteriocin β Peptide Isolated from *Lactobacillus salivarius* K4

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ABSTRACT

Salvicin K (Sal K) and antimicrobial peptide-like bacteriocin β (Alb β) peptides produced by *Lactobacillus salivarius* K4 have been reported as bacteriocins from lactic acid bacteria (LAB). Here, we aimed to characterize their activity against gram-positive bacteria, *Streptococcus* sp. TISTR 1030 and *Staphylococcus aureus* TISTR 118. The antimicrobial activity increased when Sal K and Alb β peptides were combined (MIC 3.59 μM). Results from scanning electron microscope (SEM) and transmission electron microscope (TEM) showed that the bacterial cell surface altered dramatically in the presence of the Sal K and Alb β mixture. The peptide mixture affected the bacterial membrane potential and caused cell cytotoxicity leading to the leakage of DNA and β-galactosidase. Secondary structure characterization of the peptide mixtures revealed increasing helical structure in the membrane mimicking environment. From our experiments, it can be concluded that a synergistic effect is obtained when both peptides are combined presumably through the formation of a complex. Combination of peptide exhibits more active against gram-positive bacteria than each individual peptide component.

Keywords: mode of action, bacteriocins, Salvicin K, antimicrobial peptide-like bacteriocin β, *Lactobacillus salivarius* K4

1. INTRODUCTION

Bacteriocins are ribosomal antimicrobial peptides found in a large number of bacteria, such as lactic acid bacteria (LAB), *Escherichia coli* and Archaea. Bacteriocins are considered potential next generation-peptide antibiotics as some of them target several multiple-drug resistant pathogens [1]. They are natural substances that specifically inhibit their related bacterial strains. *Lactobacillus salivarius* K4 was first isolated from chicken intestine. Characterization of bacteriocin from *Lb. salivarius* K4 reveals antimicrobial activity against *Lb. sakei* subsp. *sakei* JCM 1157^T, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124^T, *Bacillus coagulans* JCM 2257^T, *Enterococcus faecalis* JCM 5803^T, *Listeria innocua* ATCC 33090^T and *Brochotrix campestris* NBRC 11547^T [2]. Two active bacteriocin fractions were obtained from reverse-phase HPLC and designated as FK12 and FK15. Partial sequence of FK12 has 94% and 91% homology with presalivaricin B bacteriocin produced by *Lb. salivarius* M7 and bacteriocin-like prepeptide (ORF3) of *Lb. salivarius* UCC118, respectively. Amino acid sequence of FK15 shows 75% similarly to bacteriocin abp 118 α, also named Salvicin K (Sal K).

Gene structural analysis of the bacteriocin genes are characterized by polymerase chain reaction (PCR) using K4 forward and K4 reverse primers (designed from gene cluster of *Lb. salivarius* UCC 118, AF408405). Nucleotide sequencing analysis of K4 PCR product reveals three bacteriocin genes. Those three genes are presalivaricin B gene, Sal K gene and antimicrobial peptide-like bacteriocin β (Alb β) gene. The sequence alignment of Sal K and Alb β indicated that they possessed the high similarity to the well-characterized class-IIb bacteriocins from *Lb. salivarius* ABP 118, abp118-α and abp118-β, respectively. Similar to other class-IIb bacteriocin peptides, Sal K and Alb β are synthesized as pre-peptides containing GG-motif leader sequence at the N-terminus [3]. The cleavage of the GG-motif leader sequence of the pre-peptides is regulated by a dedicated protease. After the cleavage of pre-peptide, the active peptides will be translocated through the cell membrane [4]. Synthesized Sal K peptide inhibits growth of *E. faecalis* JCM 5803, *Lb. plantarum* ATCC 14917 and *Streptococcus* sp. TISTR 1030, while Alb β peptide shows antimicrobial activity against *E. faecalis* JCM 5803, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124, *Lb. sakei* TISTR 890, *Lb. plantarum* ATCC 14917, and *Streptococcus* sp. TISTR 1030 [5]. In this study, we demonstrated the antimicrobial potency of each individual Sal K, Alb β and mixture of Sal K and Alb β peptides against gram-positive bacteria; *Streptococcus* sp. TISTR 1030 and *S. aureus* TISTR 118. The antimicrobial activity of the peptides was determined in the form of (a) a standard MIC, (b) cytotoxicity in a bacterial cell cytotoxicity assay and (c) bacterial membrane depolarization and permeability determination. Damage to the bacterial cell envelope caused by the two-peptide mixture was observed by transmission electron microscope (TEM) and scanning electron microscope (SEM). Furthermore, we determined the peptide secondary structure by circular dichroism (CD) spectroscopy and measured the diffusion coefficient of the peptide in a micellar environment using nuclear magnetic resonance (NMR) spectroscopy.

2. MATERIALS AND METHODS

2.1 Synthetic Sal K and Alb β Peptides

Two cationic peptides; Sal K and Alb β peptides (GenBank accession number KU561065) were synthesized (Biomatik) using the amino acid sequences as previously reported [5]. The peptides were purified by

gradient HPLC (solvent A: 0.1% TFE in 100% acetonitrile and solvent B: 0.1% TFE in 100% water) with a PLRP-S 100A column and a 1.0 mL/min flow rate. The peptide mass was confirmed using mass spectrometry technique provided by the manufacturer.

2.2 Bacterial Strains

Gram-positive bacteria *Streptococcus* sp. TISTR 1030 and *Staphylococcus aureus* TISTR 118 were used as bacterial indicators in order to determine bacteriocin activity. Both gram-positive bacteria were kindly provided by Associate Professor Sunee Nitisinprasert from the Specialized Research Unit: Prebiotics and Probiotics for Health, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok, 10900, Thailand.

2.3 Minimum Inhibitory Concentration (MIC) assay

MICs of peptide against *Streptococcus* sp. TISTR 1030 and *S. aureus* TISTR 118 were determined by the broth microdilution method in a nutrient broth [6]. Briefly, the Sal K and Alb β peptide powder were dissolved in water to gain 230 μM peptide stock solution. pH was adjusted to 4.0. For the peptide mixture, 1:1 ratio of each peptide stock solution was combined to gain 115 μM mixture solution. In this experiment, all different concentrations of peptides were prepared in two-fold serial dilutions. 50 μL of each peptide dilution were added into a 96-well microtiter plate. After that, 10 μL of bacterial cell suspension containing 1×10^6 CFU/mL from a log-phase bacterial cell culture were added. To study MIC, bacterial cells and peptide solution mixture were incubated at 37 °C for 18 h. The treated bacteria, including positive and negative controls, were spread on nutrient agar plates and incubated at 37 °C for 24 h. The MIC was defined as the lowest peptide concentration that completely inhibited bacterial growth. The negative and positive controls were also prepared and compared to the treated samples. The negative control was the nutrient broth without bacterial cell or antimicrobial peptides. The positive control was the bacterial cells in nutrient broth without antibacterial peptides. The experiment was performed in triplicate.

2.4 Effect of Sal K/Alb β peptide Mixture on *Streptococcus* sp. TISTR 1030 using CellTox® Green Cytotoxicity Assay

To investigate the cytotoxicity of the peptide mixture to gram-positive bacteria, the CellTox® Green Cytotoxicity assay (Promega, USA) was used. The assay utilizes an asymmetric cyanine fluorescent dye which targets bacterial DNA. When the bacterial membrane integrity has changed, the dye can specifically bind to the DNA that leaks from the bacterial cells. The binding of the fluorescent dye and bacterial DNA results in an enhancement of the fluorescent intensity. In this experiment, *Streptococcus* sp. TISTR 1030 was used as bacterial indicator. The cells were cultured overnight in nutrient broth at 37 °C in an orbital shaker. Cells were harvested and washed with fresh nutrient broth and resuspended in fresh nutrient broth. The bacterial cells were further cultured until the OD_{600} reached 0.4-0.5, then washed twice in 0.5 mM PBS buffer, pH 7.4. The cells were diluted to an OD_{600} = 0.2 in 0.5 mM PBS buffer, pH 7.4. To determine the effect of the Sal K/Alb β peptide mixture, its activity was assessed at two different concentrations, 3.59 μM (1× MIC equivalent) and 7.18 μM (2× MIC equivalent). Fifty microliter of bacterial cell suspension (1×10^6 CFU/mL) were added to each well of a 96-well plate containing fresh nutrient broth and the peptide mixture at desired

concentrations. Fluorescent dye at the concentration of 0.1× was added to the microplate. The plate was kept at room temperature and shielded from light for 30 minutes before the measurement to minimize its impact on the fluorescence of the dye-DNA complex. Lysis buffer from the CellTox® Green Cytotoxicity assay kit was added to the cell suspension as a positive control while the cell suspension containing fluorescent dye without the peptide mixture was used as negative control. The fluorescence intensity was measured at an excitation wavelength of 510 nm and emission wavelength of 535 nm at 24 °C using Infinite F200PRO. The instrument was operated by Tecan i-control 1.7 software. The results were derived from three independent experiments. Student's t test was applied to calculate all statistical analyses, expressing as mean ±SD. Significant differences ($p < 0.05$) between means were identified using the Tukey procedures.

2.5 Effect of Sal K/Alb β Peptide Mixture on Depolarization of *Streptococcus* sp. TISTR 1030 Cell Membrane using 3, 3′-dipropylthiadicarbocyanine Iodide ($DiSC_3(5)$)

The bacterial membrane potential was measured by using the membrane potential sensitive cyanine dye, 3, 3′-dipropylthiadicarbocyanine iodide or $DiSC_3(5)$. The cationic dye can accumulate on the hyperpolarized membranes and translocate into the lipid bilayer. After membrane depolarization occurs, the dye will be released and its fluorescence can be detected. *Streptococcus* sp. TISTR 1030 was grown to the early exponential phase (OD_{600} = 0.4) in nutrient broth. The cells were harvested and washed three times with a washing buffer containing 5 mM HEPES, 20 mM glucose and 100 mM KCl, pH 7.4. *Streptococcus* sp. TISTR 1030 at the concentration of 2×10^5 CFU/mL was resuspended in reaction buffer containing 5 mM HEPES, 20 mM glucose and 100 mM KCl, pH 7.4. The cell suspension was treated+ with lysis buffer (positive control), 3.59 µM (1× MIC) of Sal K/Alb β peptide mixture or 7.18 µM (2× MIC). The negative control was prepared using reaction buffer instead of the peptide solution. The treated samples were further incubated at 37 °C for 60 min. After that, the cell samples were transferred to 1.5 mL centrifuge tubes and 5 µM of membrane-potential dye $DiSC_3(5)$ (TCI, Japan) was added. The solution was transferred to a quartz cuvette and the fluorescence intensity was measured using a luminescence spectrometer LS50B. The measurement was performed using an excitation wavelength of 622 nm, and the emission wavelength was recorded at 670 nm. The experiments were performed in triplicate. Student's t test was applied to calculate all statistical analyses, expressing as mean ±SD. Significant differences ($p < 0.05$) between means were identified using the Tukey procedures.

2.6 Detection of Cytoplasmic Permeation using 2-nitrophenyl β-D-Galactopyranoside (ONPG)

To study membrane permeability of the Sal K/Alb β peptide mixture, we decided to follow the enhancement of β-galactosidase enzyme which will be released after cytoplasmic membrane permeated. In this study, we used ONPG as a substrate for the β-galactosidase enzyme in order to monitor the release of the enzyme from damaged cells. Hydrolysis of ONPG by the enzyme releases ortho-nitrophenol, a yellow compound, which can be detected by UV-Visible spectroscopy at an absorbance of 420 nm. A change in the ortho-nitrophenol concentration implies an equivalent change

in β-galactosidase concentration. The assay method was modified from previous work [7] and the measurement was prepared as follow: 2×10^5 CFU/ml of *Streptococcus* sp. TISTR 1030 in PBS buffer pH 7.4 was treated with 3.59 µM (1× MIC) or 7.18 µM (2× MIC) of Sal K/Alb β peptides mixture. 2 mM ONPG was added to the solutions and incubated for 30 min. After that, the concentration of ortho-nitrophenol was measured by UV-Visible spectrophotometer (Cary 50 Conc, VARIAN) at the A_{420}. Lysis buffer and nutrient broth were used to replace the peptide mixture as a positive control and a negative control, respectively. The absorbance of each sample was measured in triplicate and compared to the control samples. Student's t test was applied to calculate all statistical analyses, expressing as mean ±SD. Significant differences ($p < 0.05$) between means were identified using the Tukey procedures.

2.7 Scanning Electron Microscopic (SEM) Examination of Changing of *Streptococcus* sp. TISTR 1030 Membrane Morphology

Each *Streptococcus* sp. TISTR1030 and *S. aureus* TISTR 118 starter cells were incubated in nutrient broth at 37 °C for 2 h prior to the addition of 3.59 µM of Sal K/Alb β peptide mixture. The treated cells were kept at 37 °C overnight. After incubation, samples were washed three times with 0.5× PBS buffer, pH 7.4 at room temperature before being incubated in 2% formaldehyde solution for 15 min. The samples were washed again with 0.5× PBS buffer, pH 7.4 three times. The bacterial cells were dropped on a 0.2 µm membrane filter. The bacterial cells on the membrane filter were rinsed with ethanol 12.5, 25, 50, 75, 100 % and then air dried [8]. The samples were deposited on SEM stubs and gold particles were sprayed onto the sample stubs with a Sputter Coater, Model SC7620. Bacterial cell samples were observed using a SEM, Model Quanta 450 (FEI, Netherlands) at 30 kV.

2.8 Transmission Electron Microscope (TEM) Examination of Changing of *Streptococcus* sp. TISTR 1030 Membrane Morphology

Overnight cell culture of *Streptococcus* sp. TISTR 1030 was incubated in fresh nutrient broth at 37 °C for 2 h. 3.59 µM of Sal K/Alb β peptide mixture was added to the cell suspension and incubated at 37 °C overnight. Untreated samples were prepared by culturing the cell suspension without adding antimicrobial peptide, which were then incubated at 37 °C overnight. Untreated and treated cells were harvested by centrifugation at 6000 rpm, 10 min and washed three times by 0.5× PBS buffer, pH 7.4 at room temperature. The TEM samples were prepared using adapted protocol from Hartmann *et al.* (2010), briefly, the untreated and treated cells were fixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 at 4 °C for 12 h. After that, the samples were post-fixed in 2% osmium tetroxide in distilled water at room temperature for 2 h [9]. Next, the samples were rinsed with distilled water three times and dehydrated by acetone 30, 50, 70, 90 and 100 %, then infiltrated and embedded in Spurr's resin. Finally the samples were polymerized at 70 °C for 8 h. The fixed samples were sectioned by thick section (100 nm thick) and cut with ultra microtome. Cellular membrane morphological changes were observed by TEM (HT7700, Hitachi Japan) and operated at accelerating voltages up to 80 kV.

2.9 Characterization of Secondary Structure of Sal K, Alb β and Peptide Mix using Circular Dichroism (CD) Spectroscopy

The secondary structures of Sal K and Alb β peptides were characterized in different buffer conditions, CD spectra of peptides were measured using the J-815 spectropolarimeter (Jasco, Japan). The experiment was setup to measure the peptides under three different buffer conditions. The buffers were water, 2 mM dodecylphosphocholine (DPC) and 34.7 mM sodium dodecyl sulphate (SDS). Experiments were performed in triplicate for each buffer set. A weight of 0.1 mg of peptide was dissolved in water, 2 mM DPC and 34.7 mM SDS. For the peptide mixture experiment, 0.05 mg of each peptide (Sal K: Alb β ratio = 1:1) was used. All CD experiments were performed at pH 4.0, 25 °C to maintain protonated amine form of lysine and arginine. CD spectra were measured in a 1.0 cm path length quartz cell at 25 °C. Spectra were recorded from 180-260 nm with a 50 nm/min scanning speed. Spectra that appeared at very high voltage were eliminated. The mean residue molar ellipticity, [θ] was obtained by the following equation. SELCON version 3 from CDPro package was used to analyze the secondary structure content of each peptide.

$$[\theta] = \frac{\theta \times 100 \times M}{C \times l \times n}$$

Where;

θ is the ellipticity in degrees
l is the optical path in cm
C is the concentration in mg/ml
M is the molecular mass, and
n is the number of residues in the protein

2.10 Translational Diffusion Coefficient Measurements

To determine the membrane binding ability of the Sal K and Alb β peptides, we decided to measure their diffusion coefficients in the presence of SDS and DPC micelles. The diffusion coefficients were measure using diffusion ordered spectroscopy (DOSY) experiment which based on the longitudinal eddy-current delay (LED) and bipolar LED sequences. The spectra of peptides in water and in the presence of SDS and DPC micelles were recorded. The number of scans was 32 per experiment and the pulse strength was varied from 10-95 % in 32 linear steps. The diffusion time (d20) was 250 ms and the gradient pulse (p30) was 2.5 ms. Trimethylsilyl propionate (TSP) was used as an internal standard. The spectra results were processed using the Bruker TopSpin 3.1 software. The T1/T2 analysis module was used to gain the diffusion coefficient. Percentage of peptide bound to micelles was calculated as:

$$[D_{obs} - D_{free}]*100/[D_{micelles} - D_{free}]$$

Where;

D_{free} is the diffusion coefficient of the free peptide in an aqueous sample
$D_{micelles}$ is the diffusion coefficient of the micelles
D_{obs} is the diffusion coefficient of the peptide

3. RESULTS AND DISCUSSION

3.1 Minimal Inhibition Concentration (MIC) Determination

The MIC was determined using *S. aureus* TISTR 118 and *Streptococcus* sp. TISTR 1030 as bacterial indicators. MIC is defined as the

lowest concentration of peptide required to inhibit bacterial growth. Sal K peptide at 115 μM (the highest tested concentration) showed no effect on bacterial growth of neither *S. aureus* TISTR 118 nor *Streptococcus* sp. TISTR 1030 (Table 2). In the presence of Alb β peptide, bacterial growth was inhibited at a concentration of 28.75 μM in both *S. aureus* TISTR 118 and *Streptococcus* sp. TISTR 1030. These results showed that both peptides had low antimicrobial activity compared to other published bacteriocins such as Nisin A and Vancomycin which have inhibiting concentration against *Streptococcus* sp. of 1.25 μM and 2.62 μM, respectively [25]. Interestingly, only 3.59 μM of Sal K/Alb β peptide mixture could inhibit *S. aureus* TISTR 118 and *Streptococcus* sp. TISTR 1030 (Table 2). These results implied that the two peptides had synergistic activity at a molar ratio of 1:1 and its antimicrobial potency increased approximately 8-fold compared to the individual Alb β peptide. The result from this study indicates that these bacteriocins require the concomitant dosing of Sal K and Alb β peptides for bactericidal effect. This finding is in accordance with the observation that two-peptide bacteriocin displays higher antimicrobial activity when combined [10]. Similar results have also been reported using two peptide bacteriocins from closely related strain, Lb. salivarius UCC118 [11], Lactococcin G [12], Lactococcin Q [13] and plantaricin J/K [14]. The interaction between Sal K and Alb β peptides is approximately 1:1 ratio which is consistent with the fact that are produced by the same promoter as the other two-peptide bacteriocins [12]. However, our result showed different from pervious study [5]. This may be due to a 10 fold higher bacterial cell concentration in previous experiments. The higher initial bacterial concentration might have resulted in a higher survival rate which negated the effect of the peptide synergism.

Table 1. Amino acid sequence, molecular weight, purity and number of amino acid of Sal K and Alb β peptides.

Peptide	Sequence (a.a.)[a]	Molecular weight (g/mol)[b]	Purity (%)[c]	No. of a.a.
Sal K	KRYPNCTGKF LGGLAKGAAL GAISGGGVPG AVIGGNIGMV AGAISCL	4346.18	86.55	47
Alb β	KNGYGGSGIR WVHCGAGIVS GALMGSIGGN AWGAVAGGIS GGIKSCR	4448.11	84.87	47

[a] amino acid

[b] Molecular weight determined by mass spectroscopy

[c] H_2O:Acetonitrile = 4:1

Table 2. Antimicrobial activity of Sal K, Alb β and Sal K/Albβ mixture against bacterial indicator *S. aureus* TISTR 118 and *Streptococcus* sp. TISTR 1030.

Peptide	*S. aureus* TISTR 118	*Streptococcus* sp. TISTR 1030
	MIC (μM)	MIC (μM)
Sal K	>115	>115
Alb β	28.75	28.75
Sal K/Alb β mixture	3.59	3.59

3.2 Effect of Sal K/Alb β Peptide Mixture on *Streptococcus* sp. TISTR 1030 using CellTox® Green Cytotoxicity Assay

The cytotoxicity was measured using the asymmetric cyanine fluorescent dye placed in the buffer. The dye specifically targets bacterial DNA. When bacterial membrane integrity is compromised, bacterial DNA will be released, which will bind to the dye resulting in enhancement of fluorescence intensity. The assays showed clear enhancement of fluorescence intensity for treated samples with 73.9 % and 90.5 % for the 3.59 μM and 7.18 μM peptide treated samples, respectively (Figure 1). The cytotoxicity of the peptide(s) against gram-positive bacteria *S. aureus* is consistent with previous results such as Polybia-MPI and its analogs, [7] as well as OaBac5-mini [15].

3.3 Membrane Polarization Effect of Sal K/Alb β Peptide Mixture on *Streptococcus* sp. TISTR 1030

In order to confirm the ability of the peptide mixture to depolarize the bacterial membrane, the effect of the membrane-potential sensitive cyanine dye, $DiSC_3(5)$ was considered. The fluorescence signal decreases as the dye partitions to the surface of polarized cells. Membrane depolarization prevents partitioning and the bound dye will be released into the media. Thus, as expected, a high fluorescence intensity was observed in the positive control of *Streptococcus* sp. TISTR 1030 incubated with the dye in the presence of the lysis buffer for complete lysis, while the negative control (no peptide mixture) produced a very low fluorescence intensity (Figure 2). Moreover, the results showed that the fluorescence intensity increased by 62.4 % for the 3.59 μM peptide mixture and 65.05 % for the 7.18 μM sample. This result indicated that Sal K/Alb β peptide mixture caused a depolarization of the *Streptococcus* sp. TISTR 1030 membrane. Even though the exact mode of action of these peptides cannot be confirmed, the results here suggested that the peptides disrupt the cytoplasmic membrane which is similar to that previously observed bacteriocin peptides such as Nisin [23], Gramicidin S [9] and PGLa [9].

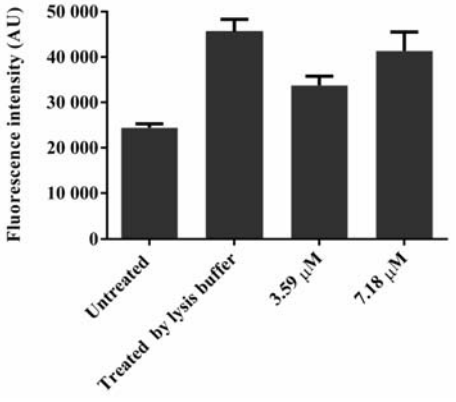

Figure 1. Effect of Sal K/Alb β peptide mixture towards the gram-positive bacteria, *Streptococcus* sp. TISTR1030 using CellTox® green cytotoxicity assay. The excitation and emission wavelengths were 510 and 535 nm, respectively. The data were analyzed by t-test which compared to the untreated sample (0 μM peptide mixture), $p < 0.05$ (*), mean ±SD (n=3).

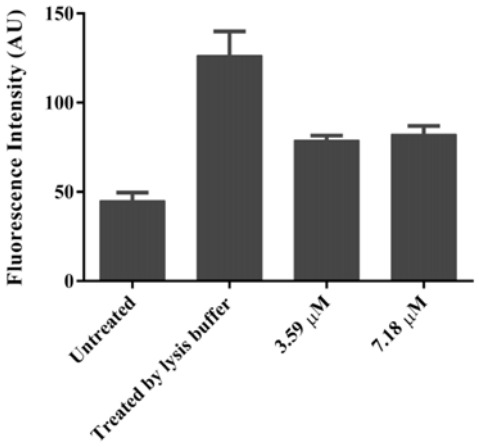

Figure 2. Bacterial membrane depolarization assay on *Streptococcus* sp. TISTR 1030 by $DiSC_3(5)$. The lysis buffer was used as positive control and the negative control was cell suspension without antimicrobial peptide treatment. The fluorescence was measured at 622 nm as excitation wavelength and emission wavelength was 670 nm. The data were analyzed by t-test which compared to the untreated sample (0 μM peptide mixture), $p < 0.05$ (*), mean ±SD (n=3).

3.4 Detection of Cytoplasmic Permeation using 2-nitrophenyl β-D-Galactopyranoside (ONPG)

To study the change in membrane permeability induced by the Sal K/Alb β peptide mixture, the release of β-galactosidase from bacterial cells was assessed. The assay utilized the detection of the extracellular enzyme (β-galactosidase) in buffer via the turnover of the colorless substrate ONPG to ortho-nitrophenol, which is quantifiable via UV-Vis spectroscopy. The result showed that β-galactosidase was released from bacterial cells after treatment with Sal K/Alb β peptide mixture (Figure 3). The level of ortho-nitrophenol was low in the negative control sample while the amount of the compound increased to approximately 53.7 % and 87.5 % in the presence of 3.59 μM and 7.18 μM of peptide mixture, respectively. This indicated that Sal K/Alb β peptide mixture perturbed the plasma membrane of *Streptococcus* sp. TISTR 1030 and resulted in the release of cytoplasmic macromolecules such as the β-galactosidase enzyme. The leakage of macromolecules such as enzymes following the treatment of Sal K/ Alb β mixture is very unusual. This result indicates that the bacteriocins from *Lb. salivarius* K4 seem to have an additional mode of actions compared to other bacteriocins in this class. Other two-bacteriocin components in previously studied, including lactacin F [17], lactococcin M [18] plantaricin A system [19] and lactococcin G [12] have been shown to cause efflux of intracellular potassium, dissipation of proton motive force and hydrolysis of internal ATP in susceptible bacteria.

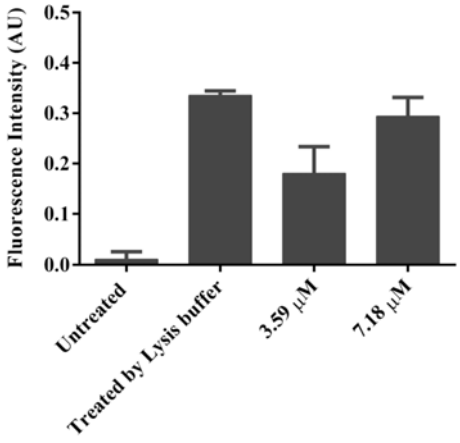

Figure 3. Determination of cytoplasmic permeabilization of *Streptococcus* sp. TISTR 1030 by ONPG assay. The ortho-nitrophenol compound was measured by UV-Visible spectroscopy method at the absorbance of 420 nm. The data were analyzed by t-test which compared to the untreated sample (0 μM peptide mixture), $p < 0.05$ (*), mean ±SD (n=3).

3.5 Morphological Changes of *Streptococcus* sp. TISTR 1030 Observed by Scanning Electron Microscopic (SEM) After Sal K/Alb β Mixture Treatment

To observe the morphological change in the gram-positive bacteria cell wall, *Streptococcus* sp. TISTR 1030 and *S. aureus* TISTR 118 cells were incubated with the peptide mixture. The results after overnight culture showed that the bacteria in the untreated sample had a normal round shape and intact cell surfaces (Figure 4A). Morphological changes were observed in the treated sample, for instance, damaged bacterial cell walls (Figure 4B), pore formation (Figure 4C and 4D), dense cells (Figure 4E), membrane debris (Figure 4F) and intracellular exposure (Figure 4G). Several distinct signs of the bacterial cell lesion are clearly observed in SEM photographs. Moreover, burst and deep craters in bacterial cell wall were noticed. Partially and completely lysed cells were observed by SEM which indicates a mechanical rupture of the cell membrane and cell wall [9].

3.6 Morphological Changes of *Streptococcus* sp. TISTR 1030 Observed by Transmission Electron Microscopy (TEM) After Sal K/Alb β Mixture Treatment

Morphological changes of *Streptococcus* sp. TISTR 1030 cell upon the treatment of Sal K/Alb β peptide mixture were followed by TEM. Intact cell walls and normal spherical shapes were observed in the untreated samples (Figure 5A and 5B). However, the bacterial cells changed their cell morphology after treating with the peptide mixture (Figure 5C and 5D). Moreover, empty spaces in the broken cells were observed with intracellular components protruding from the damaged area around the cell wall as well as a leakage of cytoplasm (Figure 5E, 5F and 5G). Some cells lacked extensive regions of the cell wall while the septum in the center of the cell differs from normal cell septa (Figure 5H).

It appeared that the combination of Sal K and Alb β peptides inhibits cell wall synthesis and causes thinning of the *Streptococcus* sp. TISTR 1030 cell wall. This thinning effect has been observed in the study of MSI-78 antimicrobial peptide using atomic force microscopy (AFM), CD and nuclear magnetic resonance spectroscopy (NMR) to investigate the effect of MSI-78 antimicrobial peptide upon phospholipid bilayers. The binding of the MSI-78 amphipathic peptide helices with helical axis parallel to the membrane surface leads to membrane thinning [20]. Some studies on antimicrobial peptides revealed that receptors on lipid bilayer are the target of cationic peptides. In 2014, Kjos *et al.* showed that the UppP protein which is involved in peptidoglycan synthesis is one of the targeted receptors on the cell membrane of *S. pneumoniae*. Nevertheless, Kjos *et al.* (2014) also showed that the interaction of Lactococcin G peptide and UppP protein can inhibit the growth of non-sensitive *S. pneumoniae* [21]. Moreover, studies of mesentaricin Y105-resistant mutant *L. monocytogenes* also showed decreased expression of the *mptACD* gene that encodes a membrane transport protein, permease [22]. Lipid II is also one of the targets for nisin. It has been proposed that the nisin peptide docks tightly with lipid II and may facilitate the membrane pore formation process [23]. Lacticin Q is found to induce high-level membrane permeability without specific receptor requirement [24]. Our results show that the combination of Sal K and Alb β

peptides targets the cell membrane of gram-positive bacteria as observed in many other two-peptide bacteriocins, e.g. lactococcin G and plantaricin J/K [14] and cationic antimicrobial peptides such as SMA29 and OaBac5 (ovine-derived antimicrobial peptides) [15], Gramicidin S and PGLa [9].

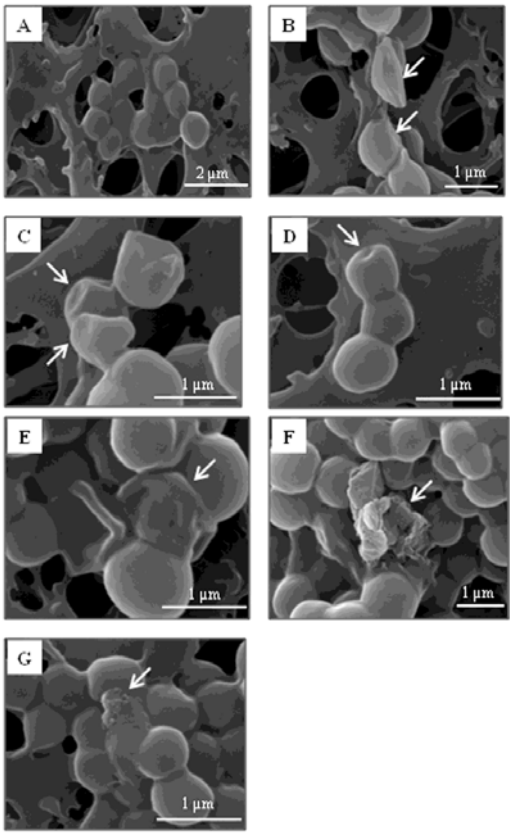

Figure 4. SEM photograph of *Streptococcus* sp. TISTR 1030 cells treated by 3.59 µM Sal K/Alb β peptide mixture (A) Untreated *Streptococcus* sp. TISTR1030 cells, (B) damaged cells, (C and D) pore formation on membrane of the treated cells, (E) dense cell, (F) debris cell and (G) exposure of intracellular component. White arrow indicates the morphological alteration of the bacterial cells.

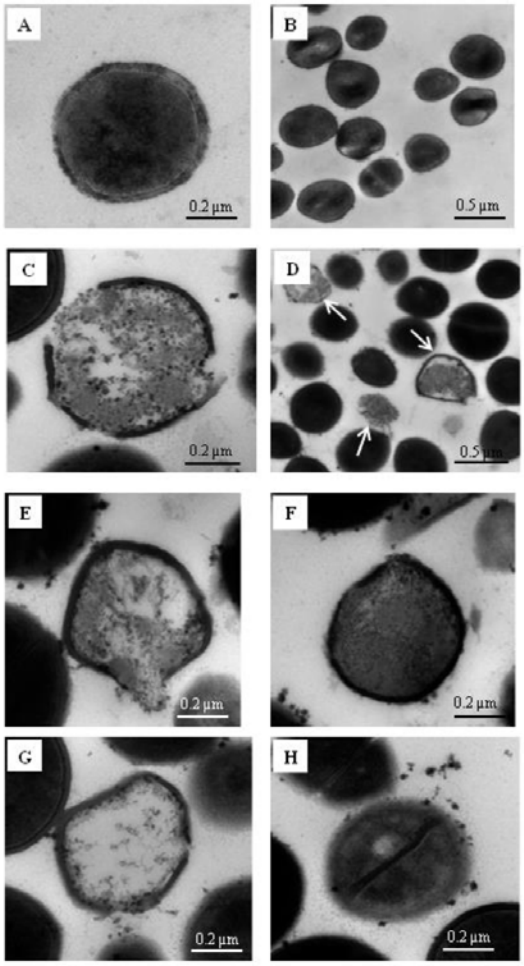

Figure 5. TEM micrograph of untreated and treated *Streptococcus* sp. TISTR1030 by 3.59 µM Sal K/Alb β peptide mixture. (A and B) untreated cells, (C and D) membrane damage, (E, F and G) Protruding of intracellular components and empty space inside the damaging cell, (H) treated cell with abnormal septum.

3.7 Structural Characterization of Sal K, Alb β and Peptide Mixture using CD Spectroscopy

Sal K and Alb β peptides were characterized in terms of their secondary structure using CD spectroscopy. Peptides

were dissolved in buffers and characterized in different environments. The percentage of secondary structure content was calculated using SELCON 3 from the CDPro package which is shown in Table 3. In the water, the individual Sal K and Alb β peptides showed peaks in negative values at 220 nm which are consistent with the CD pattern of small helical content structure (Figure 6). This result contrasts to the previous CD report for the Sal K and Alb β peptides in which a preference for a random-coil structure was observed for each individual peptide at pH 9.0 [5]. It should be noted that our experiments were done at acidic pH (pH 4.0) which is considered to be a more appropriate pH for these peptides in the lactic acid bacteria. Therefore, our data should represent the physiological structures of these peptides which tend to have helical structure in water.

Secondary structural characterization of the peptide mixture in water at pH 4.0 showed peaks in further negative values at 220 nm which may imply that the peptide mixture displays more helical structure in water at acidic pH. This suggests that the peptide mixture adopts greater helical secondary structure when compared to the individual Sal K and Alb β peptides under the same condition in water at pH 4.0.

Table 3. Percent of secondary structure component of Sal K, Alb β and peptide mix in water, 2mM DPC and 34.7 mM SDS, pH 4. 0 calculated by SELCON3 in CDPro package.

Peptide	Secondary structure content	Water	2 mM DPC	34.7 mM SDS
Sal K	Helix	24.9	46.3	80.8
	Sheet	15.7	13.7	0.4
	Turn	26.5	16.9	5.3
	Unstructured	41.7	24.7	14.4
Alb β	Helix	29.4	47.1	87.4
	Sheet	19.3	22	0
	Turn	19.5	16.6	5.7
	Unstructured	28.2	18.5	15.5
Peptide Mix	Helix	33.5	43.3	82
	Sheet	20.6	13.7	0
	Turn	21.3	19.7	16
	Unstructured	29.6	26.1	19.6

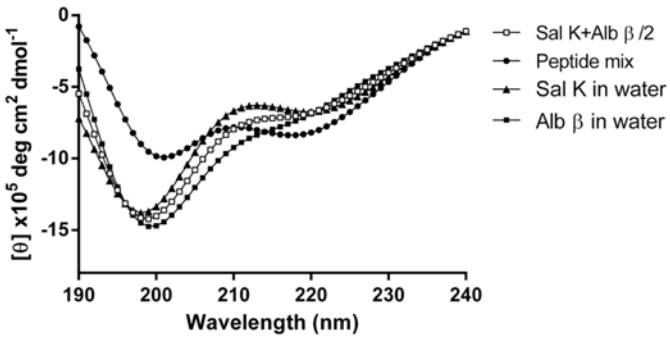

Figure 6. CD spectra of Sal K (▲), Alb β (■) and peptide mixture (•) in water at acidic pH (pH 4.0). CD spectra of Sal K +Alb β /2 (□), a combination of Sal K and Alb β CD spectrum, showed different spectra from the peptide mixture indicating the interaction between Sal K and Alb β

Furthermore, to understand the structure of these peptides in action against other bacteria cell membranes, micelles mimicking cell membranes were investigated. When dissolved the peptides in 2 mM DPC and 34.7 mM SDS buffers, the helical content of both individual peptides increased by approximately 40 % to 80 % in DPC and SDS micelles, respectively (Table 3). The percent of helical content is stronger when the secondary structure of the peptides was determined in 34.7 mM SDS. This also indicates that these cationic peptides in acidic pH prefer to interact with anionic micelles (SDS) compared to the zwitterionic micelles (DPC). It should be noted that the helical content of the peptide mixture in the presence of micelles seems to be lower than the helical content observed in each individual peptide. This observation may imply that the individual Sal K and Alb β peptides have stronger interaction with the micelles than between the peptide complex and micelles.

3.8 Partition Coefficient of Sal K and Alb β Peptides in the Presence of DPC and SDS Micelles

To confirm the binding between peptides and micelles, partition coefficient by NMR experiments in DPC and SDS micelles was performed. The experiments were performed at 25 °C pH 4.0. The diffusion coefficient of peptides in the presence of 80 mM DPC decreased when compared to the diffusion coefficient of the peptides in water (D_{free}), this result indicated that both peptides bound to DPC micelles. Nevertheless, the partition coefficient revealed 100 % binding between DPC micelles to both peptides (Table 4). Sal K peptide in the presence of 140 mM SDS showed a decreased diffusion coefficient when compared to the diffusion coefficient of the free peptide in aqueous solution. The partition coefficient of Sal K peptide in the presence of SDS micelles showed 62.5 % binding while the Alb β showed 96.7 % binding. This implied that both peptides bound to SDS micelles. Therefore, we can conclude that Sal K and Alb β peptides have the propensity for binding to membrane-mimicking micelles, DPC and SDS.

Table 4. Partition coefficients of Sal K peptide in DPC and SDS micelles at 25 °C.

		DPC	SDS
Sal K	D_{free} ($\times 10^{-7}$ cm^2s^{-1})	15.45±0.3	15.45±0.3
	D_{obs} ($\times 10^{-7}$ cm^2s^{-1})	10.00±0.1	11.10±0.4
	$D_{micelles}$ ($\times 10^{-7}$ cm^2s^{-1})	10.80±0.1	8.50±0.1
	Bound[a] (%)	100	62.5
Alb β	D_{free} ($\times 10^{-7}$ cm^2s^{-1})	16.63±0.5	16.63±0.5
	D_{obs} ($\times 10^{-7}$ cm^2s^{-1})	9.19±0.3	7.80±0.1
	$D_{micelles}$ ($\times 10^{-7}$ cm^2s^{-1})	10.8±0.1	7.50±0.3
	Bound[b] (%)	100	96.7

[a] the percentage of Sal K peptide bound to micelles was calculated as $(D_{obs}-D_{free})\times 100/(D_{micelles}-D_{free})$, where D_{free} is the diffusion coefficient of the free Sal K peptide in water sample. $D_{micelles}$ is the diffusion coefficient of the micelles and D_{obs} is the diffusion coefficient of the peptide that measured in the present of micellar system.

[b] the percentage of Alb β peptide bound to micelles was calculated as $(D_{obs}-D_{free})\times 100/(D_{micelles}-D_{free})$, where D_{free} is the diffusion coefficient of the free Alb β peptide in water sample. $D_{micelles}$ is the diffusion coefficient of the micelles and D_{obs} is the diffusion coefficient of the peptide that measured in the present of micellar system.

4. CONCLUSIONS

It can be concluded from the range of experimental studies reported herein that the synergistic effect of both peptides is important for their action against gram-positive cocci, *Streptococcus* sp. TISTR 1030. The mixed peptide solutions could alter the cell membrane surface and membrane potential of the bacteria causing cell damage. The structural characterization of the peptides indicates that the peptide mixture binds to the cell membrane-mimicking environment. This binding increases the helical content of the peptides mixture.

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