

Antimicrobial peptides of *Lactobacillus salivarius* K4 isolated from chicken intestine

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ABSTRACT: Two cationic antimicrobial peptides, salvicin K (sal K) and antimicrobial-like bacteriocin β (alb β) from *Lactobacillus salivarius* K4 were isolated from chicken intestine. In the active form, each peptide contains 47 amino acids with 2 cysteine residues. The antimicrobial activity showed that sal K could inhibit the food pathogens *Enterococcus faecalis* JCM 5803, *Lb. plantarum* ATCC 14917, and *Streptococcus* sp. TISTR 1030. The alb β showed 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. A high concentration of these bacteriocins was found to cause hemolysis in rat erythrocytes. Each peptide was predicted to have a mixed secondary structure of α -helix, β -sheet, and random coil structure with one disulphide bond. Two antimicrobial peptides were found to be unstructured in Tris-Cl pH 9. These two peptides adopted an α -helix conformation in 10% SDS micelles (to simulate the bacterial cell wall). In zwitterionic dodecylphosphocholine micelles and liposome, these two peptides adopted a β -sheet structure. The results suggest that the α -helix is an important conformation for these peptides to express their antimicrobial activity.

KEYWORDS: food pathogen, antimicrobial activity, structure and function relationship

INTRODUCTION

The production of small antibiotic peptides is a common defence strategy against bacteria displayed not only by microorganisms, but also by animals and plants¹⁻³. These antibiotics are often small cationic membrane-active compounds that form pores in the target cells, disrupting membrane potential, and causing cell death. The antimicrobial peptides (AMPs) have been reported to kill or inhibit a variety of organisms or cells, including gram-positive, gram-negative bacteria, viruses, protozoa, parasites, fungi, and cancer cells⁴⁻⁶. The AMPs can be classified into four structural groups. First, the peptides exhibit the amphipathic α -helical structure in a lipid membrane. These peptides are often unordered in solution. Second, the β -sheet peptides contain one or more disulphide bonds. The third group consists of loop-structured peptides containing one or more disulphide bonds. The fourth group comprises extended peptides with one or two amino acids predominating⁷⁻⁹. Well characterized antimicrobial peptides have a size varying from 30 to over 100 residues and consist of a wide range of different amino acids¹⁰. The mode of

action of these peptides can be categorized into two mechanisms: transmembrane pore-forming mechanisms and modes of intracellular killing^{11,12}. The transmembrane pore-forming mechanisms have been proposed for three models: barrel-stave model^{12,13}, carpet model¹⁴, and toroidal pore model¹⁵.

Bacteriocins are antimicrobial peptides synthesized by bacterial ribosomes¹⁶⁻¹⁸. They are produced by many bacterial species including several members of lactic acid bacteria (LAB). The bacteriocins from LAB have a potential use as food preservatives to substitute the chemical preservation. There are many reported examples of LAB inhibition against the spoilage and pathogenic bacteria *Pseudomonas fluorescens* and *Staphylococcus aureus*¹⁹. Two antimicrobial peptides, salvicin K (sal K) and antimicrobial-like bacteriocin β (alb β), are produced from *Lactobacillus salivarius* K4 isolated from chicken intestine. A previous report described two peptides in *Lactobacillus salivarius* K7 isolated from chicken intestine. These peptides were found to function against the food pathogens *Leuconostoc mesenteroides* and *Bacillus coagulans*²⁰. In this study, sal K and alb β were synthesized, their activities characterized, and their

structures determined under various conditions. Our results show that these cationic peptides could inhibit some food-spoilage bacteria and exhibit an α -helix structures in a membrane-mimicking environment.

MATERIALS AND METHODS

Synthetic peptides

Sal K and alb β are cationic peptides. The active forms of sal K and alb β contain 47 amino acids. These two peptides were synthesized without any modification at both N- and C-termini (AAPPTec). These peptides were confirmed by HPLC and MS analyses. The amino acid sequence of active sal K from N- to C-termini is KRYPNCTGKFLGGLAKGAALGAISGGGVPGAVIGGNIGMVAGAISCL. The amino acid sequence of active alb β from N- to C-termini is KNGYGGSGIRWVHCGAGIVSGALMGSIGGNAWGAVAGGISGGIKSCR.

Bacterial strains

To determine the antimicrobial activities of peptides, food-spoilage bacteria were used. The bacteria included lactic acid bacteria group and gram-positive group. The lactic acid bacteria were *Enterococcus faecalis* JCM 5803, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124, *Lactobacillus sakei* TISTR 890, *Lactococcus cremoris* TISTR 1344, *Lactobacillus plantarum* ATCC 14917, and *Streptococcus* sp. TISTR 1030. The gram-positive bacteria were *Brochothrix campestris* NBRC 11547, *Listeria innocua* ATCC 33090, *P. fluorescence* TISTR 358, *Staphylococcus aureus* TISTR 118, and *Bacillus coagulans* TISTR 1447. These bacteria were obtained from the Department of Biochemistry, Faculty of Science, Kasetsart University.

Antimicrobial activity

The antimicrobial activity of each peptide was tested by a broth microdilution assay⁹. The lactic acid bacteria were grown overnight in de Man, Rogosa, Sharpe (MRS) broth at 250 rpm, 30 °C. The gram-positive bacteria were grown overnight in tryptic soy broth (TSB) at 250 rpm, 30 °C. The overnight cultures were diluted in either MRS or TSB to a final concentration of 10⁷ cells/ml. A stock of each peptide was prepared in water at 500 μ g/ml and diluted twofold in serial dilution up to 15.6 μ g/ml. Then 10 μ l of each concentration of peptide was aliquoted into microtitre plates. For synergism experiment, sal K and alb β were mixed in a ratio of 1:1 in each concentration. The 100- μ l diluted overnight cultures were then added into the plates. The samples were left overnight at

room temperature and the culture turbidity visually examined. MICs are reported as an interval from *a* to *b*, where *a* is the highest concentration tested at which bacteria are still growing and *b* is the lowest concentration that causes complete growth inhibition.

Haemolysis activity

Haemolysis in the microtitre plate was determined. The 2% of rat erythrocytes was prepared in PBS buffer. A stock of each peptide was prepared in water at 500 μ g/ml and diluted two-fold serial dilution to 15.6 μ g/ml in microtitre plates. Each well containing 10 μ l of each concentration of peptides mixed with 100 μ l of diluted rat erythrocytes was left at room temperature overnight. For synergism experiment, sal K and alb β were mixed in a ratio of 1:1 in each concentration. The 0.1% triton X-100 was used as positive control for 100% haemolysis. The absorbance at 540 nm was monitored by NanoDrop 2000 UV-Vis spectrophotometer (Thermoscientific) to measure the release of haemoglobin. The percentage of haemolysis was calculated as $(A_s - A_b)/(A_p - A_b)$, where A_s = A540 sample, A_b = A540 PBS buffer, and A_p = A540 positive control.

Structural characterization by circular dichroism

Circular dichroism (CD) spectra of peptides were measured by J-715 spectropolarimeter (Jasco, Japan). The concentrations of sal K and alb β were prepared at 0.1 mM in various conditions which were (i) Tris-Cl pH 9, (ii) 10% SDS pH 9, (iii) 2 mM dodecylphosphocholine (DPC) pH 9, and (iv) liposomes pH 8. The spectra were measured at room temperature in 1-mm path length quartz cell. The scanning speed was 50 nm/min from 190–260 nm. Each spectrum was the average of three scans. The percentage of the secondary structures was analysed by SELCON Version 3²¹.

Sequence alignment and structural prediction

Amino acid sequences of sal K and alb β were aligned in the antimicrobial peptide database aps.unmc.edu/AP/main.php²² and analysed for protein structure prediction in www.predictprotein.org²³. The sequence with the highest similarity for each peptide was shown. The percentages of secondary structure, disulphide bond, and transmembrane region were predicted.

RESULTS

Antimicrobial activity of sal K and alb β

The active forms of sal K and alb β were synthesized. Each peptide was diluted to concentration in the range

Table 1 MICs of bacteriocins sal K and alb β .

Bacterial strain	MIC ($\mu\text{g/ml}$)	
	sal K	alb β
<i>E. faecalis</i> JCM 5803	15.6–31.3	15.6–31.3
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> JCM 6124	> 500	62.5–125
<i>Lactobacillus sakei</i> TISTR 890	> 500	250–500
<i>Lactococcus cremoris</i> TISTR 1344	> 500	> 500
<i>Lactobacillus plantarum</i> ATCC 14917	15.6–31.3	250–500
<i>Streptococcus</i> sp. TISTR 1030	15.6–31.3	15.6–31.3

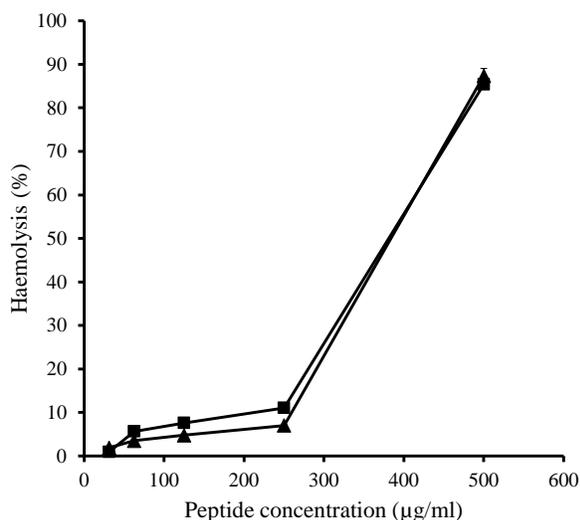


Fig. 1 Haemolysis activities of sal K and alb β were tested against rat erythrocytes. Triton-X 100 (0.1%) was used as positive control for 100% haemolysis and PBS buffer for negative control.

15.6–500 $\mu\text{g/ml}$. Table 1 shows the antimicrobial activities (MICs) of each peptide against 6 strains of lactic acid bacteria. Sal K shows activities against *E. faecalis* JCM 5803, *Lb. plantarum* ATCC 14917, and *Streptococcus* sp. TISTR 1030, while alb β expresses activities against *E. faecalis* JCM 5803, *Leuconostoc mesenteroides* subsp. *mesenteroides* JCM 6124, *Lb. sakei* TISTR 890, *Lb. plantarum* ATCC 14917, and *Streptococcus* sp. TISTR 1030. Synergistic activity of these peptides was not found. For activity of peptides against gram-positive bacteria, only alb β had activity against *Brochothrix campestris* NBRC 11547. Both peptides showed MICs > 500 $\mu\text{g/ml}$ against other gram-positive bacteria.

Haemolytic activity of sal K and alb β

Haemolytic activity of sal K and alb β shows similar results (Fig. 1). The concentrations of these peptides were found up to 500 $\mu\text{g/ml}$ to obtain 90% of haemol-

ysis. The LC_{50} values of sal K and alb β were 371.8 and 366.2 $\mu\text{g/ml}$, respectively. Synergistic activity of these peptides was not found.

Structural characterization by circular dichroism

CD spectra of sal K and alb β were measured in different conditions: (i) 20 mM Tris-Cl pH 9, (ii) 10% SDS, (iii) 2 mM DPC, and (iv) liposome. Sal K and alb β structures were found unordered in the Tris-Cl buffer solution. In the presence of anionic micelles, 10% SDS, sal K, and alb β showed α -helical structure, but in 2 mM DPC or liposomes they presented β -sheet structure (Fig. 2). The percentage of the secondary structure of each peptide was calculated by SELCON3 (Table 2).

Sequence alignment and structural prediction

The amino acid sequences of sal K and alb β are aligned in the antimicrobial peptide database. The highest similarity percentage between sal K and salivaricin P is 62%. For alb β , the alignment result shows that sakacin P has the highest similarity percentage at 42% (Fig. 3). The amino acid sequences of sal K and alb β were submitted to www.predictprotein.org to identify the predicted structure. The results show that sal K contains a mixed structure in different percentages of α -helix, β -sheet, and random coil of 26%, 19%, and 55%, respectively. One disulphide bond is predicted from Cys6 and Cys46. The transmembrane region at C-terminal was found from Ala18 to Leu47 (Fig. 4). For alb β , it contains mixed structure of α -helix, β -sheet, and random coil of 17%, 34%, 49%, respectively. One disulphide bond is detected between Cys14 and Cys46. The transmembrane region is found from Gly15 to Gly41.

DISCUSSION

Antimicrobial peptides show a wide range of activities, including antibiotic activity against gram-positive and gram-negative bacteria, antifungal activity, and antiviral activity. LAB are common bacteria that

high density of negative charge and are predominantly composed of phosphatidylglycerol, cardiolipin, a phosphatidylserine. The mammalian membranes are composed of zwitterionic phospholipids, phosphatidylethanolamine, phosphatidylcholine and sphingomyelin⁴. In this study, the anionic micelles created by 10% SDS represent the prokaryotic cell membrane whereas DPC is the zwitterionic micelles representing an eukaryotic cell membrane^{24,25}. The structure of these two peptides are found as helical structure in 10% SDS micelles which corresponds to the protein prediction result with the residue 9–20 of sal K and the residue 18–25 of alb β . The sequence similarity between sal K and salivaricin P was 62%. Since the structure of salivaricin P has not yet been characterized, the 3D structure of either sal K or salivaricin P needs to be further investigated to explain the mechanism. The alignment result shows that alb β and sakacin P have a 42% sequence similarity. The sakacin P was produced in *Lactobacillus sake* Lb674. The residue 18–25 of alb β shows sequence similarity to the residue 18–33 of sakacin P (Fig. 3). This region of sakacin P was revealed as helical structure in a micellar environment by NMR technique²⁶. This implied that at the residue 18–25, alb β can adopt a helix conformation similar to that of sakacin P. The tertiary structure of alb β however needs to be examined to confirm the results of structural prediction. Other cationic peptides such as halocidin⁹ and indolicidin¹⁰ also show α -helical structure in a micellar environment suggesting that this structure may be employed during pore formation. In halocidin, α -helical structure was found when this peptide was in TFE and 10% SDS. In DPC, zwitterionic micelle, sal K and alb β performed structure majority in β -sheet. These results imply that the active conformation of sal K and alb β is α -helical structure. Since these two peptides can release the haemoglobin, they may be either α -helix membrane pore formers or membrane disrupter as detergent-like model. The pore-forming peptides or proteins such as, colicin^{27–30}, Cry toxin^{31–33}, haemolysin E³⁴ can disturb the transmembrane potential, pH gradient, and osmotic regulation of the cells which eventually leading to cell lysis. In the detergent-like model, protein will aggregate on the membrane until they could disrupt the structure of the lipid. Cyt1A is one of the proteins whose function has been hypothesized in detergent-like model³⁵. The transmembrane region of these two peptides comprise hydrophobic residues as predicted from the results. Sal K amino sequence was similar to salivaricin P³⁶. This peptide is also found in *Lactobacillus salivarius* DPC 6005 which is a different strain from this study.

The salivaricin P shows antimicrobial activity against *E. faecalis* similar to that of sal K. The salivaricin P also exhibits activity against *Staphylococcus aureus* and *Streptococcus thermophilus*. The antimicrobial activity of sakacin P is found against *E. faecalis*, *Leuconostoc mesenteroides*, and *Lb. sakei*³⁷ similar to alb β . In contrast to alb β , sakacin P shows no activity against *Lb. plantarum*. No synergism of sal K and alb β was observed in the antimicrobial activity or haemolysis assay. These results suggest that these two peptides may perform independent functions.

CONCLUSIONS

This study reveals the structure and function of sal K and alb β from *Lactobacillus salivarius* K4 isolated from chicken intestine. The two antimicrobial peptides can adopt an unordered structure or α -helix for antimicrobial activity. These bacteriocins may be applied to food to protect against food pathogen bacteria. The details of their structures are needed to be further investigated by NMR to better explain their mechanisms of action.

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